CYTOPLASMIC POLYADENYLATION OF MATERNAL TRANSCRIPTS BY WISPY IN DROSOPHILA MELANOGASTER

by Jun Cui

CYTOPLASMIC POLYADENYLATION OF MATERNAL TRANSCRIPTS BY WISPY IN DROSOPHILA MELANOGASTER

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Gametogenesis and early embryogenesis in many animal species often occur in the context of little or no new transcription. Instead, they rely on the translation of pre-existing mRNAs that had been synthesized and stockpiled earlier in gametogenesis. Translation of these mRNAs must be repressed during their synthesis and deposition, and then be activated later. One mechanism to regulate the translation of maternal mRNAs is to control the length of their 3' poly(A) tails through cytoplasmic polyadenylation. Studies in *C. elegans* identified a cytoplasmic poly(A) polymerase (PAP) GLD-2 that, in complex with an RNA-binding protein GLD-3, extends poly(A) tails of stored maternal mRNAs during oogenesis.

Here, I identified the two GLD-2 cytoplasmic PAPs in *Drosophila melanogaster*. Using yeast two-hybrid assays I showed that both can interact with the Drosophila GLD-3 (Bic-C). I showed that one Drosophila GLD-2 PAP is expressed in the female germline, and the other in testes. I focused my subsequent research on the female-expressed GLD-2 PAP, which is encoded by the *wispy* gene.

I showed that WISP activity is essential for polyadenylation of mRNAs during two key transitions: oocyte maturation and egg activation. *wisp* null mutations block development during the egg-to-embryo transition. Female meiosis resumes and completes in a proportion of the activated fertilized eggs laid by *wisp* mutant females, but pronuclear migration does not occur. Using a PCR-based poly(A) tail (PAT) assay

to test the polyadenylation of candidate mRNAs, I found that *wisp* activity is required to extend the poly(A) tails of several maternal mRNAs in maturing oocytes or in early embryos. WISP-dependent transcripts during oocyte maturation include the *dmos* mRNA. WISP-dependent transcripts during egg activation include the *bicoid, Toll,* and *torso* mRNAs.

To identify other maternal mRNAs that are regulated by WISP-mediated cytoplasmic polyadenylation, I used microarray analyses to globally identify mRNAs whose poly(A) tail lengths depend on active WISP. I found that thousands of maternal mRNAs are not polyadenylated in the absence of WISP function. My results indicate that WISP-mediated cytoplasmic polyadenylation is a major mechanism to regulate maternal mRNAs in oocyte development and early embryogenesis in Drosophila.

BIOGRAPHICAL SKETCH

Jun was born and grew up in Shanghai, the largest city located on the east coast of the mainland China. He attended Shibei High school where he got interested in science, especially in chemistry, and later on decided to study science in college. During his senior year in high school, he was selected by an undergraduate research program at Fudan University and started his college life before finishing the last year in high school. Jun chose biology over chemistry as his major in college because he found understanding the nature of life was more exciting than finding the composition of the world. After four years of college, he felt he needed to know more about biology. So he left his home town and moved to Ithaca, a gorgeous city which sits on the southern shore of Cayuga Lake. Jun joined the Wolfner lab at Cornell University to study developmental biology, focusing on the regulation of maternally supplied messenger RNAs in Drosophila. Protein products of these mRNAs are essential for egg development. After Cornell, Jun will move to Stanford University and start his postdoctoral training in the Reijo Pera lab working on human germ cell development.

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I finally would like to extend my heartful gratitude to all my friends and family members. Most especially to my parents who always understand me and believe in me. Their patient love enabled me to complete this PhD study.

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LIST OF ABBREVIATIONS

AD	Activation domain
APC/C	Anaphase promoting complex/cyclosome
ARE	(A+U)-rich element
BD	Binding domain
bcd	*bicoid*
Bic-C	*Bicaudal-C*
cAMP	Cyclic adenosine 5’monophophate
cort	*cortex*
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CPSF	Cleavage and polyadenylation specificity factor
CSF	Cytostatic factor
cycA	*cyclin A*
cycB	*cyclin B*
eIF	Eukaryotic translation initiation factor
ERK	Extracellular signal-related kinase
gnu	*giant nuclei*
GO	Gene Ontology
grp	*grapes*
GSK-3	Glycogen synthase kinase 3
GST	Glutathione-S-transferase
hb	*hunchback*
hrg	*hiiragi*
IP	Immunoprecipitation

JNK	Jun N-terminal kinase
KD	Kinase domain
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mfr	*misfire*
mh	*maternal haploid*
mor	*moira*
MTOC	Microtubule organizing center
mtrm	*matrimony*
nos	*nanos*
NRE	NOS response element
osk	*oskar*
PABP	Poly(A)-binding protein
PAP	Poly(A) polymerase
PAT	PCR-based poly(A) test
PBD	Polo-binding domain
pim	*pimples*
PKA	Protein kinase A
plu	*plutonium*
png	*pan gu*
prg	*prage*
pum	*pumilio*
RNP	Ribonucleoprotein
SCMC	Subcortical maternal cortex
smg	*smaug*
snky	*ms(3)sneaky*

SRE	SMG response element
sra	*sarah*
ssm	*sesame*
stg	*string*
Tl	*Toll*
tor	*torso*
tPA	Tissue plasminogen activator
TUB	Tubulin
UTR	Untranslated region
wisp	*wispy*
Ya	*Young arrest*

CHAPTER 1

INTRODUCTION

Regulation of gene expression in a living animal is a very delicate system. An important part of this system is the regulation of transcription, which determines when and where a certain gene can be made into messenger RNAs. However, transcription is just the start of gene expression, and more layers of regulation will act on the transcripts before they can be translated into protein products. The posttranscriptional mechanisms regulate every aspect of the mRNA, such as its stability, localization and translation. These mechanisms are usually mediated by the cis-acting elements within the transcript and trans-acting factors such as proteins present in the cell. These factors can either activate or repress translation of the transcript in different contexts.

The role of posttranscriptional regulation is particularly crucial when there is no or limited transcription during some developmental processes such as very early embryogenesis. Maternal mRNAs are deposited into eggs during oogenesis. The transcriptional machinery is silent in late oogenesis and early embryogenesis, until the activation of the zygotic genome (Tadros and Lipshitz, 2005). As so many maternal mRNA molecules are accumulated in the oocyte, it is critical to control precisely the translation of these pre-existing mRNAs both temporally and spatially. Cytoplasmic polyadenylation is a key regulatory mechanism that controls the translation of maternal mRNAs during the development of germ cells (Richter, 2007).

What is cytoplasmic polyadenylation?

The initial polyadenylation of an mRNA molecule occurs when the mRNA is transcribed in the nucleus. The nuclear pre-mRNA processing adds a poly(A) tail of

about 200 adenosine residues, according to the animal species, to the 3' end of the mRNA (Colgan and Manley, 1997). Polyadenylation of a newly synthesized transcript is a two-step coupled reaction involving cleavage and polyadenylation (Millevoi and Vagner, 2010). Most mRNAs contain a polyadenylation signal sequence, the hexanucleotide AAUAAA (Hex). Transcription goes beyond this element, generating a pre-mRNA longer than the mature form. A protein complex, the cleavage polyadenylation specificity factor (CPSF), then recognizes the Hex element and cleaves the transcript at about 10-30 nucleotides downstream of the element. Meanwhile, CPSF recruits a nuclear poly(A) polymerase (PAP) to form the poly(A) tail on the mRNA. Nuclear PAPs are very conserved proteins and are widely found in different tissues in different animals (Colgan and Manley, 1997).

The function of the poly(A) tail is believed to protect the mRNA from degradation (Garneau et al., 2007). In somatic cells, a long poly(A) tail recruits the poly(A)-binding protein (PABP) which can protect the transcript from exonucleases; shortening of the poly(A) tail usually indicates the beginning of mRNA decay. The poly(A) tail is also believed to have a role in translation activation, but how that happens is still not yet clear. Studies have suggested that the poly(A) tail can help to initiate translation by interacting with the mRNA's 5' cap structure (m^7GpppN) (Preiss and Hentze, 1998; Tarun and Sachs, 1995). A long poly(A) tail recruits the poly(A) binding protein (PABP), which interacts with the translation initiation factor eIF4G and probably stabilizes its association with eIF4E (Craig et al., 1998; Tarun and Sachs, 1996; Wakiyama et al., 2000). This PABP-eIF4E-eIF4G complex circularizes the mRNA and probably enhances the efficiency of translation (Wakiyama et al., 2000). Thus regulating the poly(A) tail length can directly affect the stability and function of the mRNA.

After mRNAs are transported from the nucleus into the cytoplasm, the fates of their poly(A) tails differ in different tissues. In somatic cells, poly(A) tails are slowly shortened while the mRNAs are actively translated (Gray and Wickens, 1998). In specific cell types, such as oocytes, in which translation of many mRNAs is tightly regulated, poly(A) tails on these regulated mRNAs are dramatically shortened to about 20 to 40 adenosine residues (Huarte et al., 1992). This results in retention of the mRNAs in a translationally dormant state until late in egg development or fertilization when the poly(A) tail of these mRNAs are extended (Kim and Richter, 2006; Pique et al., 2008; Sarkissian et al., 2004). Subsequent poly(A) tail lengthening is an important form of translational regulation that activates the translation of specific mRNAs and results in rapid changes in the amounts of their protein products (Barkoff et al., 2000; Gebauer et al., 1994; Mendez et al., 2000a). This type of poly(A) tail control is driven by cytoplasmic factors. It is known as cytoplasmic polyadenylation, and is distinct from nuclear polyadenylation.

Key factors involved in cytoplasmic polyadenylation

Although much of the mechanism of cytoplasmic polyadenylation is not yet clear, several key components of this machinery have been identified in different animals. It appears that the interaction between cis-elements within the mRNA and trans-acting protein factors is important to determine when and where a particular mRNA can be polyadenylated. Cis-acting elements in the transcripts are believed to mark specific mRNAs that are subject to cytoplasmic polyadenylation (Richter, 2007). Protein factors are then recruited to these elements to facilitate the poly(A) lengthening of the mRNA. The 3' untranslated regions (3'UTRs) of the transcripts are often the site of these regulatory elements. So far two such elements required for cytoplasmic polyadenylation have been best characterized.

The first specific element, the cytoplasmic polyadenylation element (CPE), is required for polyadenylation of a number of mRNAs, including *cyclin B1* and *mos*, during oocyte maturation and early embryo development in vertebrates (Barkoff et al., 2000; Groisman et al., 2000; Mendez et al., 2000a; Paris and Richter, 1990). A protein that binds to the CPE, the CPE binding protein CPEB, was isolated by affinity chromatography using the CPE sequence as the substrate (Hake and Richter, 1994). CPEB binds directly to the CPE element in the 3'UTR of maternal mRNAs. These mRNAs undergo strong cytoplasmic polyadenylation (Stebbins-Boaz et al., 1996). Studies in mouse confirmed the role of CPEB in cytoplasmic polyadenylation (Racki and Richter, 2006; Tay and Richter, 2001).

Studies in Xenopus identified a second element within the mRNA, the nuclear polyadenylation signal, Hex, that is also required for cytoplasmic polyadenylation (Fox et al., 1992; McGrew et al., 1989; McGrew and Richter, 1990; Simon et al., 1992). Disruption of the Hex element in an mRNA's 3'UTR represses its cytoplasmic polyadenylation and translation in the cytoplasm. Hex is recognized by CPSF protein complex (Bilger et al., 1994; Dickson et al., 1999). CPSF can strongly stimulate polyadenylation in the presence of CPEB because CPEB physically interacts with a CPSF subunit, which is believed to help the recruitment of CPSF to the CPE-containing mRNA (Dickson et al., 1999; Mendez et al., 2000b).

The poly(A) polymerase is another important component of cytoplasmic polyadenylation. This enzyme was unknown until shortly before the start of my thesis research. It had been thought that the canonical nuclear PAPs were used again in cytoplasmic polyadenylation (Juge et al., 2002; Kashiwabara et al., 2000). However, the recent finding of a family of new poly(A) polymerase suggested that cytoplasmic polyadenylation had its own catalytic enzyme. The first member of the family, GLD-2, was identified in *Caenorhabditis elegans* (Wang et al., 2002). Immunostaining showed

that GLD-2 is predominantly found in the cytoplasm of germ line and early embryo. Recently a second cytoplasmic PAP, GLD-4, was found in *C.elegans.* It acts in parallel to GLD-2 to promote meiotic progression (Schmid et al., 2009). GLD-2 proteins have also been found in other animals. The Xenopus GLD-2 homolog was found to be in the same complex with other cytoplasmic polyadenylation components, such as CPEB and CPSF (Barnard et al., 2004). Mammals also have GLD-2 homologs (Kwak et al., 2004), but it is still not clear if these homologs play a role in polyadenylation. Disruption of the mouse GLD-2 does not affect polyadenylation (Nakanishi et al., 2007).

GLD-2-type PAPs belong to the same nucleotidyl transferase family as the nuclear PAPs (Wang et al., 2002). However the nuclear and cytoplasmic PAPs are quite different. The most obvious difference in protein structure is that GLD-2 does not contain any RNA binding motif (Wang et al., 2002). This suggests that recruitment of GLD-2 to RNA must be mediated by a separate RNA binding protein. Yeast two-hybrid experiments showed that *C. elegans* GLD-3, a RNA binding protein, can interact with GLD-2 (Wang et al., 2002). In vitro nucleotidyltransferase assays further showed that purified GLD-2 and GLD-3 can form a dimer with poly(A) polymerase activity, which supports the model that these two proteins function as a complex in promoting polyadenylation (Figure 1.1). A second *C.elegans* GLD-2 binding protein, RNP-8, was recently reported (Kim et al., 2009). RNP-8 associated with GLD-2 promotes the sperm fate, while the GLD-3/GLD-2 complex promotes the oocyte fate.

Activation of cytoplasmic polyadenylation in Xenopus oocyte maturation

Cytoplasmic elongation of poly(A) tails was first discovered in marine animals (Wilt, 1973), and it was found to regulate translation of maternal mRNAs after fertilization (Rosenthal et al., 1983). Since then many studies mainly using Xenopus

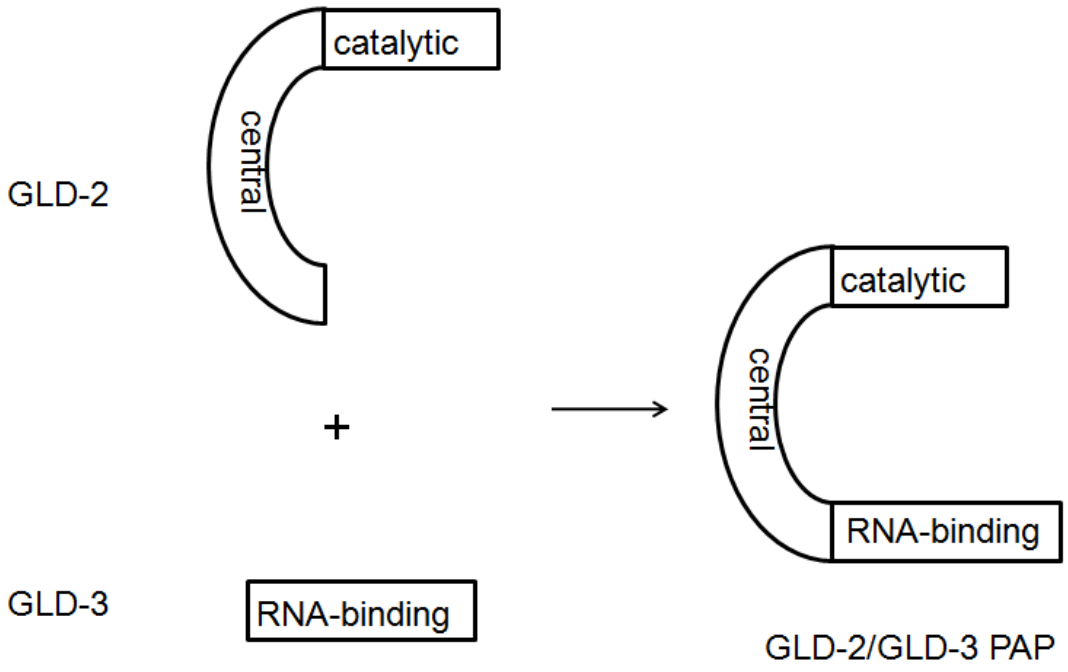


Figure 1.1 Model for GLD-2/GLD-3 PAP enzyme in *C.elegans*. GLD-2 has a catalytic domain (catalytic) and a central domain (central). GLD-3 has a RNA recognition motif (RNA-binding). GLD-2 and GLD-3 form a heterodimer with poly(A) polymerase activity. (Adapted from Wang, 2002)

oocyte extracts have revealed a complicated regulatory machinery of poly(A) tail control during oocyte maturation.

Oocyte development in Xenopus arrests at two points. The first arrest is at prophase I. Upon a hormonal signal, oocytes re-enter meiosis in a process also known as oocyte (meiotic) maturation. Prophase I arrest is maintained by a high level of intracellular cyclic adenosine 5′monophosphate (cAMP) which activates protein kinase A (PKA) (Newhall et al., 2006; Tsafriri et al., 1996). PKA phosphorylates and activates the inhibitory kinases, Wee1 and Myt1, which lead to the inhibitory phosphorylation of CDC2 (Leise and Mueller, 2002). PKA also phosphorylates and represses the CDC25 phosphatase whose activity is to remove inhibitory phosphorylation of CDC2 (Duckworth et al., 2002). Both pathways result in a lower level of CDC2 activity, which prevents entry into meiosis. Progesterone induces a decrease in cAMP levels, which leads to the inactivation of PKA. This thus activates CDC2 activity, which drives metaphase entry (Eyers et al., 2005).

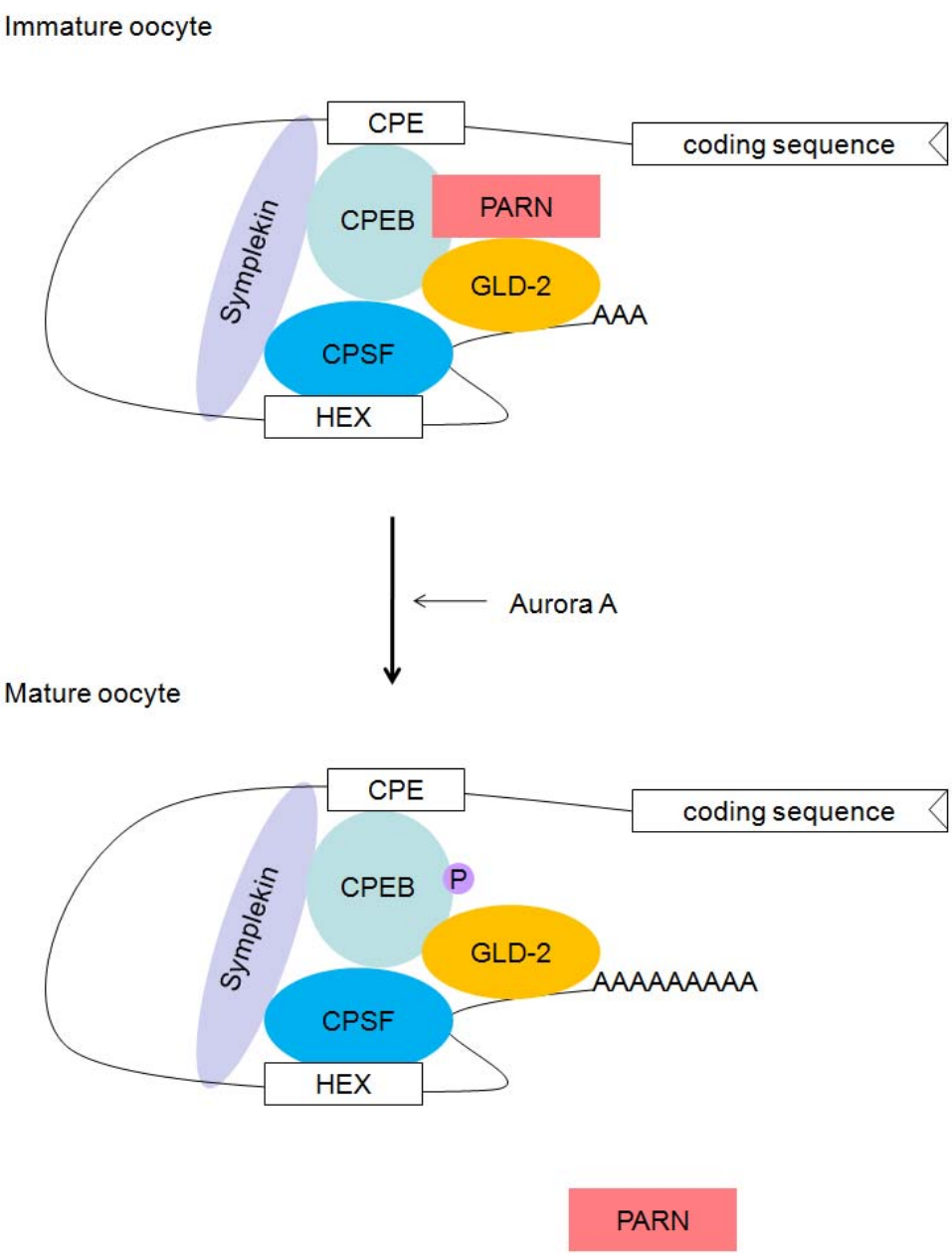
Oocyte maturation is complete when meiosis reaches a second arrest point, metaphase II (MII) in Xenopus. Mature oocytes arrest there until fertilization. In vertebrate oocytes, the MII arrest is maintained by the activity called cytostatic factor (CSF). Mos is a component of the CSF activity (Sagata et al., 1989) which is believed to activate a downstream kinase p90Rsk through the mitogen-activated protein kinase (MAPK) cascade (Bhatt and Ferrell, 1999; Gross et al., 1999). A crucial component of CSF is identified recently in Xenopus as Emi2/XErp1, an inhibitor of the anaphase promoting complex (APC/C), which connects p90Rsk to cell cycle regulation to maintain the CSF arrest (Schmidt et al., 2005; Tung et al., 2005).

Oocyte maturation depends on the translational activation of a series of protein factors. Evidence has been shown that translation of these factors is regulated by temporal control of poly(A) tails before and after oocyte maturation. Maternal mRNAs

are deadenylated in early oocytes to repress translation. An early study on the poly(A) tail length of tissue plasminogen activator (tPA) mRNA in the early mouse oocytes (Huarte et al., 1992) revealed that this mRNA acquires a long poly(A) tail when transcribed in the nucleus. Its poly(A) tail is shortened after being transported into the cytoplasm to repress translation of tPA, and the shortening depends on the RNA's CPE. A similar finding was seen for the Xenopus *cyclin B1* mRNA. Its poly(A) tail undergoes CPE-mediated shortening in early oocytes (Kim and Richter, 2006). A poly(A) ribonuclease (PARN) in Xenopus oocytes is responsible for the deadenylation of this mRNA (Copeland and Wormington, 2001).

In immature oocytes, a cytoplasmic polyadenylation complex is associated with mRNAs with CPE elements. This protein complex consists of CPEB, CPSF and a scaffold protein Symplekin (Barnard et al., 2004). GLD-2 is also associated with this complex but the presence of PARN antagonizes the function of GLD-2 (Kim and Richter, 2006). Poly(A) tail length depends on a balance between the activity of PARN and GLD-2. In early oocytes when CPEB is dephosphorylated, PARN works more efficiently. The net outcome is shortening of the tail (Kim and Richter, 2006). The PARN-CPEB association is regulated by Aurora A kinase (Sarkissian et al., 2004). In immature Xenopus oocytes, Aurora A is phosphorylated and its activity is inhibited by glycogen synthase kinase 3 (GSK-3) when PKA is active. When progesterone triggers Xenopus oocyte maturation, GSK-3 activity is decreased, due to the decrease in cAMP levels. This situation activates Aurora A, which in turn phosphorylates CPEB at Serine 174 and triggers the disassociation of PARN from CPEB (Kim and Richter, 2006; Mendez et al., 2000a). Phosphorylation of CPEB increases its affinity for CPSF, which helps to stabilize CPSF in the cytoplasmic polyadenylation complex (Mendez et al., 2000b). All these help to activate GLD-2 poly(A) polymerase and result in the elongation of poly(A) tails (Figure 1.2).

Figure 1.2 Model of CPE-mediated polyadenylation in Xenopus. CPEB binds CPE element within the 3'UTR of the mRNA and forms a complex with CPSF, which recognizes the Hex (AAUAAA) element. Other factors are also recruited to the complex including symplekin (a scaffold protein), PARN deadenylase and GLD-2 poly(A) polymerase. In immature Xenopus oocytes when CPEB is dephosphorylated, PARN is anchored to CPEB and antagonizes the function of GLD-2, which results in deadenylation of the mRNA. Upon oocyte maturation, the kinase Aurora A is activated to phosphorylate CPEB, which triggers the disassociation of PARN and stabilizes CPSF. All these help to activate GLD-2 and result in elongation of poly(A) tails. (Adapted from Kim and Richter, 2006)


Immature oocyte
CPE
coding sequence
Symplekin
CPEB
PARN
GLD-2
AAA
CPSF
HEX
Aurora A
Mature oocyte
CPE
coding sequence
Symplekin
CPEB
P
GLD-2
AAAAAAAAA
CPSF
HEX
PARN

Cytoplasmic polyadenylation plays an important role in Xenopus meiotic progression by activating the translation of key regulators of the cell cycle such as Cylin A and Cyclin B (Stebbins-Boaz et al., 1996). This leads to the activation of M-phase promoting factor (MPF), a heterodimer of Cyclin B and CDC2, and progression of meiosis. Translation of *mos* mRNA is also activated by poly(A) tail elongation (Gebauer et al., 1994; Mendez et al., 2000a). Mos protein, a mitogen-activated protein (MAP) kinase kinase kinase, helps to activate MPF during metaphase entry and maintain the meiosis II arrest later on.

Polyadenylation of different mRNAs happens in a temporal order during meiotic progression. Some CPE-containing mRNAs, such as *cyclin B2 and cylin B5*, are polyadenylated and activated much earlier than other mRNAs. The timing of polyadenylation of a mRNA is determined by the combination of the number and placement of CPEs in the 3'UTR (Pique et al., 2008). A deadenylation mechanism also contributes to the temporal control of cytoplasmic polyadenylation. The interaction between C3H-3, a RNA binding protein, and (A+U)-rich elements (AREs) in the 3'UTR of certain mRNAs recruits a deadenylase to these mRNAs. This recruitment leads to a temporal repression of both cytoplasmic polyadenylation and translation of these mRNAs (Belloc and Mendez, 2008).

Correlation between polyadenylation and translation

Polyadenylation is believed to be generally correlated with translation rate. Shortening of the poly(A) tail represses translation and is a strategy used to keep maternal mRNAs untranslated (Huarte et al., 1992). Oocyte maturation triggers cytoplasmic polyadenylation and translational activation of maternal mRNAs (Atkins et al., 2004; Mendez et al., 2000b; Stebbins-Boaz et al., 1996). It is not yet clear how translation is activated. One experiment suggest that it is the process of poly(A) tail

elongation, rather than the poly(A) tail itself, that activates translation: injection of a depolyadenylated mRNA which contains a functional cytoplasmic polyadenylation element into fertilized Xenopus eggs can induce translation; but the same mRNA cannot be translated if its cytoplasmic polyadenylation element is disrupted, even after a long poly(A) tail was added to the mRNA before the injection (Simon et al., 1992). This suggests that the recruitment of the cytoplasmic polyadenylation machinery, instead of the poly(A) tail, is required to activate translation.

Although the poly(A) tail is not required for activation of translation, it can help to initiate translation and enhance the translation rate. A long poly(A) tail recruits PABP, which stabilizes the interaction between the translation initiation factor eIF4G and eIF4E (Craig et al., 1998; Tarun and Sachs, 1996; Wakiyama et al., 2000). This PABP-eIF4E-eIF4G complex circularizes the mRNA and probably enhances the efficiency of translation (Wakiyama et al., 2000). A long poly(A) tail can also help to destabilize translational repressors from the mRNA. Studies in Xenopus suggest that some maternal mRNAs are "masked" in the immature oocyte by an inhibitory protein Maskin (Stebbins-Boaz et al., 1999). Maskin is recruited to CPE-containing mRNAs by interacting with CPEB. Maskin can also interact with eIF4E through a motif that mediates the eIF4E-eIF4G interaction, thus Maskin prevents eIF4E from binding with eIF4G and inhibits the translation initiation (Cao et al., 2006; Cao and Richter, 2002). It is believed that when CPEB is phosphorylated and the poly(A) tail elongates during oocyte maturation, recruitment of PABP helps to stabilize the eIF4E-eIF4G association and to displace Maskin from eIF4E.

Poly(A) tail control during Drosophila oogenesis and early embryogenesis

Drosophila oocyte development starts in the germarium (Spradling, 1993). Cystoblasts derived from the division of a germline stem cell undergo four rounds of

division in the gemarium to form a cyst of 16 interconnected germ cells. Fifteen of the cells become nurse cells. The remaining one cell arrests in prophase I of meiosis and will develop into the oocyte. Nutrients are transported from the nurse cells into the oocyte though the ring canals that connect all the sixteen cells (Serbus et al., 2005; Theurkauf, 1994). Oogenesis has 14 developmental stages and the oocytes grow dramatically in size by accumulating a pile of maternal molecules. Meiotic maturation occurs after stage 13 and female meiosis progresses to metaphase I where it arrests. The arrest results from the kinetochore forces created by chiasmata (Joyce and McKim, 2009; McKim et al., 1993).

Mature (stage 14) oocytes are arrested at metaphase I in meiosis until egg activation. Egg activation prepares the egg for subsequent development. Unlike vertebrates and marine invertebrates in which egg activation is triggered by fertilization (Ducibella et al., 2006; Parrington et al., 2007), in Drosophila egg activation is triggered by mechanical stress experienced by the egg when it passes through the female reproductive tract during ovulation (Heifetz et al., 2001; Horner and Wolfner, 2008). Egg activation triggers the resumption and completion of female meiosis. In wild-type Drosophila eggs, when both meiotic divisions are completed, the chromosomes of the four meiotic products decondense (Foe et al., 1993). The most centrally located female meiotic product usually becomes the female pronucleus and migrates toward the male pronucleus. The male-provided centrosome divides and assembles the first zygotic spindle (Callaini and Riparbelli, 1996). Two zygotic nuclei form at the end of the first mitotic division. The fertilized eggs of Drosophila and some other insects lay eggs that exhibit syncytial divisions, in which the nuclei undergo rapid S and M phases but cytokinesis does not occur. During this time, protein concentration gradients formed along the embryo axes are important in the determinating of the developmental pattern of the embryo.

Most aspects of Drosophila oocyte development and early embryogenesis are under the control of maternally supplied transcripts. Poly(A) tail length control is one mechanism that regulates these maternal transcripts. Deadenylation is used in many cases to repress the translation of maternal mRNAs while activation of these mRNAs often correlates increases in poly(A) tail length. Maternal transcripts have short poly(A) tails in early oocytes and poly(A) tail elongation occurs during oogenesis.

Cyclin protein levels are tightly regulated in early oocytes to control the number of cystoblasts (Lilly et al., 2000). Overexpression of Cyclin A in the cystoblasts results in an extra round of cell division to produce 32 cells in the cysts. Low cyclin levels are also required to maintain the prophase I arrest in oocytes (Sugimura and Lilly, 2006). Translational repression of cyclin mRNAs is partially mediated by poly(A) tail shortening. The CCR4 deadenylase, encoded by the *twin* gene, is responsible for the poly(A) tail shortening of both *cyclin A* and *cyclin B* mRNAs (Benoit et al., 2005; Morris et al., 2005). Oocytes produced by *twin* mutant females show defects in germ cell number and oocyte specification during early oogenesis (Morris et al., 2005), suggesting that proper poly(A) tail shortening is important for oocyte development.

Cytoplasmic polyadenylation of maternal mRNAs occurs during late oogenesis and in early embryos. Elongation of the poly(A) tails is important to activate the translation of cyclin mRNAs. During oocyte maturation, PAN GU (PNG) kinase activates both polyadenylation and translation of *cyclin A* mRNA, which promotes cell cycle progression into prometaphase (Vardy et al., 2009). Poly(A) tail elongation of *cyclin B* mRNA also occurs during oocyte maturation. About 100 to 120 adenosine residues are added to its mRNA (Benoit et al., 2005; Vardy and Orr-Weaver, 2007). This polyadenylation is mediated by the ORB protein, the Drosophila homolog of CPEB, and is believed to activate the translation of *cyclin B* for the exit from prophase

I arrest. Later on, egg activation triggers another round of polyadenylation of *cyclin B* mRNA (Vardy and Orr-Weaver, 2007). This polyadenylation depends on both ORB and PNG functions. Poly(A) tail elongation of *cyclin B* mRNA in the embryo probably helps to maintain the Cyclin B level when the protein is rapidly degraded by the APC/C during syncytial divisions.

Translational repression and activation of several Drosophila maternal mRNAs that determine embryo polarity are also correlated with changes in poly(A) tail length of the mRNAs. For example, some maternal transcripts such as *bicoid*, *Toll*, and *torso,* are not translated in mature oocytes (Casanova and Struhl, 1989; Driever and Nusslein-Volhard, 1988; Hashimoto et al., 1988). Egg activation triggers polyadenylation and translation of these mRNAs (Salles et al., 1994).

It is not clear how egg activation triggers polyadenylation, but a recent study suggests that the calcium signaling pathway is required to activate polyadenylation upon egg activation (Horner et al., 2006). In Drosophila the *sarah* gene, which encodes the Drosophila calcipressin that inhibits the calcium-dependent phosphatase calcineurin, is required for egg activation. Polyadenylation and translation of *bicoid* mRNA does not occur in embryos from *sarah* mutant mothers. Completion of progression of female meiosis might also be needed for triggering polyadenylation upon egg activation. The *cortex* gene, which encodes an activator of the APC/C that degrades Cyclin A, is required for progression in female meiosis (Pesin and Orr-Weaver, 2007). *bicoid* mRNA does not undergo poly(A) tail elongation in embryos produced by *cortex* mutant mothers (Lieberfarb et al., 1996).

Maternally supplied *hunchback* mRNA is another example of poly(A) tail regulation. Maternal *hunchback* mRNA has a short poly(A) tail of about 30 nucleotides in the early oocytes, and no *hunchback* translation is detected there (Wreden et al., 1997). Its poly(A) tail elongates to about 70 nucleotides in early

embryos; this correlates with the increase in its translation. However, *hunchback* mRNA is not translated in the posterior of the embryo. This repression is mediated by PUMILIO (PUM) protein, a RNA binding protein, which recognizes the NOS response element (NRE) of *hunchback* mRNA 3'UTR. PUM recruits NOS to the *hunchback* mRNA, which in turn recruits the CCR4 deadenylase and results in translational repression of *hunchback* mRNA (Kadyrova et al., 2007).

Besides its role in translation, poly(A) tail is also thought to regulate maternal mRNA stability. Maternal transcripts are degraded during early embryogenesis (Tadros et al., 2003). This degradation is required to allow zygotic transcripts to assume control of embryo development. Degradation of maternal transcripts in the Drosophila embryos is mediated by a RNA binding protein SMAUG (SMG), which recruits the CCR4 deadenylase to specific maternal transcripts (Semotok et al., 2005; Tadros et al., 2007). This deadenylation machinery is responsible for the elimination of over 1600 maternal transcripts, which is critical for the maternal-to-zygotic transition (Tadros et al., 2007).

Knowns and unknowns of the cytoplasmic polyadenylation machinery in Drosophila

Very little is known about the mechanism of cytoplasmic polyadenylation in Drosophila. So far only a handful of components involved in the cytoplasmic polyadenylation machinery are identified. One protein of interest ORB, the Drosophila homolog of CPEB, is required for oocyte development and is involved in translation regulation during oogenesis (Castagnetti and Ephrussi, 2003; Lantz et al., 1994). For example, ORB mediates the polyadenylation and translational activation of the posterior determinant *oskar* in the oocyte (Castagnetti and Ephrussi, 2003; Chang et al., 1999).

Another protein of interest is Bic-C, the Drosophila homolog of GLD-3, which is only expressed in the germline cells and is required during early oogenesis (Mahone et al., 1995). It has been shown recently to be involved in regulating the poly(A) tail length of several mRNAs during oogenesis (Chicoine et al., 2007). Bic-C seems to have dual functions at different stages during oogenesis. In early stages of oogenesis, it functions as a repressor of polyadenylation by recruiting the CCR4 deadenylase complex to the mRNAs. The poly(A) tails of several mRNAs, including *Bic-C* RNA, are elongated in *Bic-C* mutants. But late in oogenesis, after stage 9-10, the poly(A) tail of Bic-C mRNA is decreased in *Bic-C* mutants, indicating that Bic-C stimulates polyadenylation at these late stages.

A missing piece of the puzzle was the poly(A) polymerase involved in cytoplasmic polyadenylation in Drosophila. There was evidence suggesting that the canonical nuclear PAP encoded by the *hiiragi (hrg)* gene can play a role in polyadenylation during oogenesis (Juge et al., 2002; Tadros et al., 2007; Vardy and Orr-Weaver, 2007). For example, HRG can interact with ORB genetically in regulating the poly(A) tail length of *osk* mRNA in the ovary. Overexpression of the *hiiragi* gene in embryos can also increase the poly(A) tail length of *bcd* and *rp49* mRNAs. However, it is not very clear if these effects in the *hiiragi* mutant were due to its role in nuclear polyadenylation. It is also not clear if GLD-2 proteins are involved in cytoplasmic polyadenylation in Drosophila germline development. In this dissertation, I focused my study on the two Drosophila GLD-2 homologs, *wispy (wisp)* and *gld-2*.

Dissertation outline

I present in this dissertation an investigation of cytoplasmic polyadenylation of maternal transcripts in the Drosophila female germline focusing on the cytoplasmic

polymerase. I have identified two GLD-2-type poly(A) polymerases in Drosophila. My analysis of the WISP protein, the GLD-2 expressed in female germline, shows that it acts to mediate cytoplasmic polyadenylation of maternal transcripts during oocyte maturation and egg activation. The poly(A) tails of many maternal transcripts are shortened in WISP-deficient eggs. My data suggest that WISP-dependent cytoplasmic polyadenylation is a general mechanism in the temporal regulation of maternal transcripts. This work identifies a key player in the regulatory polyadenylation machinery and helps to dissect the mechanism that controls maternal mRNAs.

In Chapter 2, I mapped the *wisp* mutations and identified the molecular lesions in the *wisp* alleles. I show that the *wisp* gene encodes a Drosophila GLD-2 protein and is specifically expressed in the female germline cells. I also show that Drosophila has a second GLD-2 which is expressed in the testis.

In Chapter 3, I report data demonstrating that WISP is required to polyadenylate and activate maternal mRNAs during both oocyte maturation and egg activation. Eggs lacking WISP function are abnormal in female meiosis with defective microtubule structures, and they arrest early in embryo development. I show that *dmos* mRNA is polyadenylated during oocyte maturation due to WISP activity and *wisp* mutants thus show low activity of the MAP kinase cascade which is downstream of Dmos in late oogenesis. Several maternal transcripts, *bicoid, Toll* and *torso,* are known to be regulated by polyadenylation in Drosophila embryos (Salles et al., 1994). My data suggest that their poly(A) tail elongation also depends on WISP function.

Chapter 4 presents the identification of other WISP regulated mRNAs using microarray analysis. My data show that WISP is a master regulator of maternal transcripts, and that poly(A) elongation is a general feature of the control of many maternal mRNAs.

In Chapter 5 I report an investigation of the function of a novel gene CG2941 in egg development. I show that maternal CG2941 mRNA is regulated by WISP-dependent polyadenylation during oogenesis. Disrupting the expression of CG2941 leads to an early arrest in embryo development, so CG2941 is needed for female fertility.

Chapter 6 discusses the findings in the study, and future directions.

At the end of the thesis, I summaried four other projects that I did during my thesis study. Appendix A describes a deletion analysis to map the essential motifs in Young Arrest (YA) that interact with Lamin Dm_0. In Appendix B, I demonstrate the interaction between Young Arrest and POLO. Appendix C is my attempt to use Drosophila as a model system in studies of human laminopathies. Appendix D contains the deficiency mapping and identification of the *prage (prg)* mutations isolated from a previous genetic screen (Tadros et al., 2003). My data indicate that CG14801 is the *prg* gene.

CHAPTER 2

IDENTIFICATION OF TWO GLD-2 PROTEINS IN DROSOPHILA MELANOGASTER[1]

2.1 Introduction

Polyadenylation of mRNA takes place initially in the nucleus of eukaryotic cells, but maintenance and regulation of the poly(A) tail occurs in the cytoplasm (Sachs et al., 1997). Cytoplasmic polyadenylation is a key regulatory mechanism that controls the function of maternal mRNAs during the early development of Xenopus oocytes (Kadyk and Kimble, 1998; Mendez and Richter, 2001). A cytoplasmic poly(A) polymerase (PAP) called GLD-2, first identified in *Caenorhabditis elegans*, functions within a protein complex to facilitate cytoplasmic polyadenylation (Barnard et al., 2004; Wang et al., 2002). GLD-2-type PAPs are different from canonical PAPs in three aspects: first, GLD-2s are present only in a few cell types, mostly germ cells and neurons, while canonical PAPs are ubiquitous; second, GLD-2s are only found in the cytosol while canonical PAPs are both nuclear and cytoplasmic; third, GLD-2s lack RNA binding ability while canonical PAPs can bind RNA directly. Recruitment of GLD-2 to RNA is mediated by a separate binding partner. For example *C. elegans*, two RNA binding proteins, GLD-3 (Wang et al., 2002) and RNP-8 (Kim et al., 2009), have been identified to recruit to GLD-2 to RNA.

Cytoplasmic polyadenylation factors are conserved among eukaryotes and some of these factors have also been identified to be important in Drosophila germline function. For example, Orb, the Drosophila homolog of CPEB, is necessary during

[1] Portions of this chapter were published in Cui et al. (Cui et al., 2008), and are reprinted with permission. Identification of CG5732 is part of a manuscript: Sartain, C., Cui, J., and Wolfner, M. F. (unpublished).

oogenesis (Castagnetti and Ephrussi, 2003; Lantz et al., 1994). However, it was not yet clear whether GLD-2 plays a role in Drosophila development.

In this Chapter I report the identification and cloning of the two GLD-2 genes in the Drosophila genome, *wispy (wisp)* and *gld-2*. These two GLD-2 proteins are expressed differentially in the germline cells of the two sexes. WISP is highly expressed in the female germline while *gld-2* encodes a male specific counterpart.

2.2 Materials and Methods

Drosophila stocks and complementation tests: Oregon-R was used as the wild type stock. $wisp^{41}$/FM6, $wisp^{248}$/FM6 and $wisp^{249}$/FM6 (Tadros et al., 2003) were kindly provided by W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). $wisp^{12\text{-}3147}$/FM0, described by (Brent et al., 2000), was obtained from the Bloomington Drosophila Stock Center (Indiana). P element insertion lines for CG32663, CG1886, CG10353, CG15738, CG15737, CG2467, and CG2471 used for complementation tests as well as *Df(1)RA47*/FM7c used for making $wisp^{41}$ and $wisp^{248}$ hemizygotes, were from the Bloomington Drosophila Stock Center. Males of P-insertion lines were crossed to $wisp^{41}$/+ or $wisp^{248}$/+ virgin females. Fertility of the *wisp*/P-insertion daughters of this cross was scored to assess complementation.

RNA extraction and RT-PCR: Total RNA was extracted from adult flies and 0- to 2-, 2- to 4-, 4- to 6-, or 6- to 24-hr embryos using TRIzol (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). PCR reactions were performed with a High Fidelity Kit (Roche Applied Science, Indianapolis, IN). The transcript for ribosomal protein RPL32 was used as a control for the quality and quantity of cDNAs used for RT-PCR (Fiumera et al., 2005). Primer sequences were as follows:

wisp P1 upper 5'-ACTATCGCAAGTCGGAATCG

wisp P1 lower 5'-AGTTGCGCCTATGCTCGATGGAC

wisp P2 upper 5'-TACCAGGCGCTAAACACCCAG

wisp P2 lower 5'-TTAGGCGACATGCGCTGCAG

gld-2 upper 5'-CCATTCGCAAATGAGTCCAAGAGG

gld-2 lower 5'- GGCTGGCGTGTAACCAAAGAA

rpl32 upper 5'-CCGCTTCAAGGGACAGTATC

rpl32 lower 5'-GACAATCTCCTTGCGCTTCT

Western blot analysis: Samples from ovarian oocytes and 0- to 15-min or 0- to 2-, 2- to 4-, 4-to 6-, 6- to 24-hr embryos were prepared for Western blotting and processed as in Sackton et al., (Sackton et al., 2007). A polyclonal anti-WISP antibody was generated by methods described in Ravi Ram et al., (Ravi Ram et al., 2005). Briefly, a GST (Glutathione-S-Transferase) fusion of the carboxy-terminal 411 amino acids of the predicted WISP protein was purified from *E. coli* cells and used to immunize rabbits (Cocalico Biologicals Inc., Reamstown, PA). Serum was first run through a Sepharose 4B column (Sigma) coupled with GST protein and then affinity-purified with the GST-WISP fusion protein. The method described above was also used to generate a polyclonal anti-DmGld-2 antibody by using a 329 amino acids region (residues 480-808) of the predicted protein as antigen. The purified anti-WISP antibody was used at 1:2,000 dilution and anti-DmGLD-2 was used at 1:1,000 dilution to probe Western blots. Anti-α-tubulin antibody (Sigma, catalog #T5168) was diluted 1:10,000.

Yeast two-hybrid analysis: Full length coding sequences from WISP, DmGLD-2 and Bic-C (Mahone et al., 1995; Saffman et al., 1998) were cloned into vectors of the Matchmaker yeast two-hybrid system (Clontech, Mountain View, CA). In–frame fusions of each coding region were generated in both the DNA-binding domain vector pGBKT7 and the activation domain vector pGADT7. Yeast cells co-

transformed with pGBKT7 and pGADT7 derivatives were grown on -Trp -Leu synthetic medium and tested for growth on -Trp -Leu -His and -Trp -Leu -His -Ade synthetic media.

2.3 Results and Discussion

The *wisp* gene encodes a female specific GLD-2: To identify the *wisp* gene at the molecular level, my co-authors and I carried out complementation analysis with mutations in 10F1-7, the polytene chromosome region to which *wisp* had been previously mapped (Brent et al., 2000; Tadros et al., 2003). My co-authors and I tested for complementation between *wisp* mutant alleles ($wisp^{41}$ and $wisp^{248}$) and the seven (out of 24 total) predicted genes in the 10F1-7 region for which P-element insertion lines were available. One line, P(SUPor-P)CG15737^{KG05287}, fails to complement both *wisp* alleles. The P element in this line is inserted in the coding region of CG15737, 105 base pairs downstream of CG15737's start codon according to the annotation of the Drosophila genome (http://www.flybase.org). These results suggest that CG15737 corresponds to the *wisp* gene.

According to the latest annotation of the Drosophila genome (http://www.flybase.org), CG15737 has one predicted open reading frame (ORF) that encodes a protein of 1373 amino acids. BLAST searches revealed that the predicted CG15737 protein has a consensus PAP/25A-associated domain which is conserved in poly(A) polymerases. CG15737 protein is a GLD-2-type poly(A) polymerase. Members of this family of PAPs do not contain any predicted RNA recognition motifs (Wang et al., 2002). CG15737 protein is one of the two predicted GLD-2 proteins in the Drosophila genome. The other is encoded by CG5732 (DmGLD-2). As shown in Figure 2.1, the predicted Drosophila GLD-2 protein sequences are homologous to other GLD-2 family members in other animals: CeGLD-2 in *C. elegans* (Wang et al.,

Figure 2.1 Alignment of the conserved region in GLD-2 proteins. Amino acid sequence of the predicted PAP domain of WISP (see Figure 2.2 for position) was aligned against those of GLD-2 proteins of Drosophila, *C.elegans,* Xenopus, human and mouse. Identical or similar residues between the sequences are indicated with an asterisk or a dot (or dots), respectively.

```
mGLD-2          HTHYIPDIVR-----------------------------------------CVPPLREIPL 141
hGLD-2          HTHYVPDIVR-----------------------------------------CVPPFREIAF 141
xGLD-2          QSPSPPVLKG-----------------------------------------HSSNSGDCWL 168
WISPY           HASHRPHGQMGGHAMSSYVPHRPPPPPHPSISSPNPTPVATGAGGPWYEMILPPDRYLAQ 949
DmGLD-2         LPLVVHNRYWREFFG-----------------------------------YTPADRFLLR 914
CeGLD-2         LTKILPTDNFR---------------------------------------GGRGFASP 532
                 .

mGLD-2          LEPREITLP-------EAKDKLSQQILELFETCQQQASDLKKKE-LCRAQLQREIQLLFP 193
hGLD-2          LEPREITLP-------EAKDKLSQQILELFETCQQQISDLKKKE-LCRTQLQREIQLLFP 193
xGLD-2          YDHIDTTLP-------VAKDKLSKQILDLFQALQQQVCDLKKKD-ICRAELQREIQQIFP 220
WISPY           ARNIEVTVQPEKLICMCKYDNLSAEIWKRFRGAQQTHNKFKLKMRLWRYLYLWMHQPMFE 1009
DmGLD-2         AKFVEMRRPPKVMGCKNKWDPLSLSVWKKFLESQQTRHVYKIKMRLWRAIYTVAMKN-YP 973
CeGLD-2         SPPTSLLSEP-----LSRMDVLSEKIWDYHNKVSQTDEMLQRKLHLRDMLYT-AISPVFP 586
                    .             * ** .: . .   .*     : *  :         .  :

mGLD-2          QSRLFLVGSSLNGFGARSSDGDLCLVVK--------EEPCFFQVNQKTEARHILTLVHKH 245
hGLD-2          QSRLFLVGSSLNGFGTRSSDGDLCLVVK--------EEPCFFQVNQKTEARHILTLVHKH 245
xGLD-2          QSRLYLVGSSLNGFGIRSSDADLCLVLK--------EEP----MNQNTEARHILSLLHKH 268
WISPY           RYRICLVGSTITGFGTDSSDIDMCLLPEQGVHPHQHQYHQHHHFHNEKRTEALIILTLFN 1069
DmGLD-2         RYGLYLVGSSISYFGSKCSDMDICMLACTN-------------PNIDSRMEAVYHLHVMK 1020
CeGLD-2         LSGLYVVGSSLNGFGNNSSDMDLCLMITN--------------KDLDQKNDAVVVLNLIL 632
                   : :***::. **  .** *:*::                  . . .   :  *

mGLD-2          FCTRLSGYIERPQLIRAKVPIVK--FRDKVSCVEFDLNVNNTVGIRNTFLLRTYAYLENR 303
hGLD-2          FCTRLSGYIERPQLIRAKVPIVK--FRDKVSCVEFDLNVNNIVGIRNTFLLRTYAYLENR 303
xGLD-2          FYTRLS-YIERPQFIRAKVPIVK--FRDKVSGAEFDLNVNNVVGIRNTFLLRTYAYLDKR 325
WISPY           AVLKDTEVFQDFNLIEARVPILR--FKDISNGIEVDLNFNNCVGIKNTYLLQLYAQMDWR 1127
DmGLD-2         ELLGRTNMFQDFNLIEARVPILR--FTDRCHKVEVDINFNNSVGIRNTHLLYCYSQLDWR 1078
CeGLD-2         STLQYEKFVESQKLILAKVPILRINFAAPFDDITVDLNANNSVAIRNTHLLCYYSSYDWR 692
                       .:  ::* *:***::  *        .*:* ** *.*:**.**  *:  : *

mGLD-2          VRPLVLVIKKWASHHDINDASRGTLSSYSLVLMVLHYLQTLPEP-ILPSLQKIYPESFS- 361
hGLD-2          VRPLVLVIKKWASHHQINDASRGTLSSYSLVLMVLHYLQTLPEP-ILPSLQKIYPESFS- 361
xGLD-2          VRPLVLVIKKWANHHGINDASRGTLSSYTIVLMVLHYLQTLPEP-ILPSLQRKYPECFD- 383
WISPY           TRPLVVIVKLWAQYHDINDAKRMTISSYSLVLMVLHYLQHACVPHVLPCLHSLYPEKFQ- 1186
DmGLD-2         VRPMALTVKQWAQYHNINNAKNMTISSYSLMLMVIHFLQVGASPPVLPCLHNLYPEKFG- 1137
CeGLD-2         VRPLVSVVKEWAKRKGINDANKSSFTSYSLVLMVIHFLQCGPTK-VLPNLQQSYPNRFSN 751
                .**:.  :* **. : **:*.. :::**:::***:*:**      :** *:  **: *

mGLD-2          ---TSV-QLHLVHHAPCNVPPYLSKNESSLGDLLLGFLKYYATEFDWNTQMISVREAKAI 417
hGLD-2          ---PAI-QLHLVHQAPCNVPPYLSKNESNLGDLLLGFLKYYATEFDWNSQMISVREAKAI 417
xGLD-2          ---RTM-QLHLVHQAPRNIPQFLSKNETPLGDLLLGFLKYFAVEFDWSKDVISLREAKAL 439
WISPY           ---LGQ-QDCLDLDLIEPIEPYQALNTQTLGEHLLGFFKYYS-TFDFRNFAISIRTGGVL 1241
DmGLD-2         ---LLQPNDFGYVDMNEVMAPYQSDNSQTLGDLLLSFLHYYS-VFDYGKYAISIRVGGVL 1193
CeGLD-2         KVDVRTLNVTMALEEVADDIDQSLSEKTTLGELLIGFLDYYANEFNYDRDAISIRQGRRV 811
                       :     .           :   **: *:.*:.*::  *::    **:* .  :

mGLD-2          PR-----------------------------------------------PDDMEWRNKYI 430
hGLD-2          PR-----------------------------------------------PDGIEWRNKYI 430
xGLD-2          PR-----------------------------------------------TDDYEWRNKYI 452
WISPY           PVSTCRM--------------------------------------AKSPKNDVYQWKEL 1262
DmGLD-2         PIEVCRA--------------------------------------ATAPKNDIHQWNEL 1214
CeGLD-2         ERAALAVRPKIHSNSEGDKETPPPSSSASTSSIHNGGTPGIPMHHSISNPHFWRSQWRCV 871
                                                           ..    : . :

mGLD-2          CVEEPFDGTNTARAVHEKQKFDMIKDQFLKSWQRLKNKRDLNSVLPLRAATLKR------ 484
hGLD-2          CVEEPFDGTNTARAVHEKQKFDMIKDQFLKSWHRLKNKRDLNSILPVRAAVLKR------ 484
xGLD-2          CVEEPFDGSNTARAVYEKQKFDLIRAEFLKAWVALRDNRDLYSLLPVKGIMKKMHSL--- 509
WISPY           NIEEPFDLSNTARSVYDGPTFERVKAVFLISARRLDHTLDLATIFRPIHHVPEHFPQL-Q 1321
DmGLD-2         CIEEPFDQTNTARSVYDTDTFERIKTIFVASYRRLDSTRNLSAIFEDYDGPTILMQQPSV 1274
CeGLD-2         CIEEPFTNSNTAHSIYDEMVFEAIKKAFREAHGELQHNHDLDKLMECEPIKASTTNTGAA 931
                 :****  :***:::::   *: ::  *  :   *  . :*  ::
```

2002), xGLD-2 in *X. laevis* (Barnard et al., 2004), mGLD-2 in mouse and hGLD-2 in human (Kwak et al., 2004; Kwak and Wickens, 2007). CG15737 shares 51-56% similarity in its PAP/25A-associated domain with the previously characterized GLD-2 proteins and CG5732 shares 50-57% similarity with other GLD-2 proteins. The PAP/25A-associated domains of the two Drosophila GLD-2 proteins are more conserved with 68% similarity.

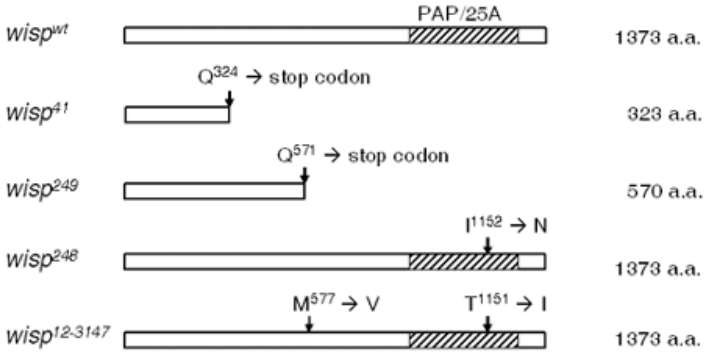
To confirm the assignment of CG15737 as *wisp*, I sequenced CG15737 in four *wisp* mutants: $wisp^{41}$, $wisp^{248}$, $wisp^{249}$, and $wisp^{12\text{-}3147}$. As shown in Figure 2.2A, all four alleles have molecular lesions in the predicted ORF of CG15737. $wisp^{41}$, $wisp^{248}$, and $wisp^{249}$ were induced in the same genetic background (Tadros et al., 2003), so the different molecular lesions found in these alleles do not represent background mutations. $wisp^{41}$ and $wisp^{249}$ are single-base-pair changes that cause nonsense mutations resulting in truncated proteins. $wisp^{248}$ is a point mutation that changes a conserved isoleucine (Ile) to asparagines (Asp) in the predicted PAP/25A domain. $wisp^{12\text{-}3147}$ has two point mutations. One is a change from threonine (Thr) to isoleucine in a residue of the PAP/25A domain adjacent to the one mutated in $wisp^{248}$; the other is a methionine- (Met) to-valine (Val) change outside the conserved domain.

I generated an antibody against WISP (see Materials and Methods). My antibody detects a protein of ~140 KDa in ovarian protein extracts of wild type and $wisp^{248}/wisp^{248}$ females, but not in ovarian protein extracts of $wisp^{41}/wisp^{41}$ and $wisp^{249}/wisp^{249}$ females, consistent with the finding that the $wisp^{41}$ and $wisp^{249}$ mutations would result in truncated proteins lacking the C-terminal epitopes recognized by this antibody (Figure 2.2B).

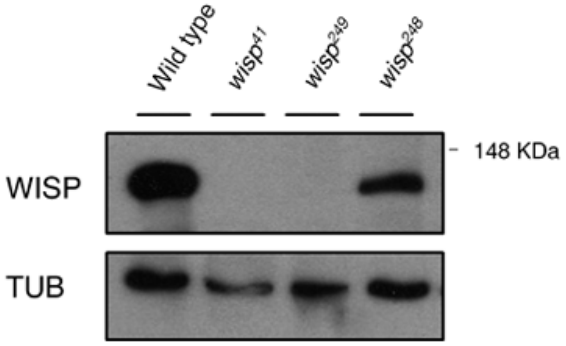
To determine the expression pattern of *wisp*, I performed RT-PCR for *wisp* mRNA using two sets of primers specific for two different regions of the predicted transcript. Transcripts of *wisp* are detected in adult flies and throughout

Figure 2.2 Wild-type and mutant protein sequences of WISP. (A) Schematic representation of *wisp* alleles. $wisp^{41}$ and $wisp^{249}$ have nonsense mutations in the coding region that result in truncated proteins of 323 a.a. and 570 a.a. respectively. $wisp^{248}$ has a point mutation at position 1152 resulting a Ile to Asp change. $wisp^{12\text{-}3147}$ has point mutations at position 577 and 1151 which result in Met-to-Val and Thr-to-Ile substitutions, respectively. (B) Western blot analysis. Ovaries were dissected from wild type, $wisp^{41}/\ wisp^{41}$, $wisp^{248}/\ wisp^{248}$ and $wisp^{249}/\ wisp^{249}$ females. Ovarian protein extracts were separated by SDS-PAGE, blotted and probed with affinity-purified polyclonal anti-WISP directed against the 411 a.a. C-term part of the protein or with anti-α-tubulin (loading control). A protein of ~140 KDa is detected in ovarian extracts of wild type and $wisp^{248}/\ wisp^{248}$ females. This band is absent in ovarian extracts of $wisp^{41}/\ wisp^{41}$ and $wisp^{249}/\ wisp^{249}$ females.

A


wisp^wt
PAP/25A
1373 a.a.
Q^324 → stop codon
wisp^41
323 a.a.
Q^571 → stop codon
wisp^249
570 a.a.
I^1152 → N
wisp^248
1373 a.a.
M^577 → V
T^1151 → I
wisp^12-3147
1373 a.a.

B


Wild type
wisp^41
wisp^249
wisp^248
148 KDa
WISP
TUB

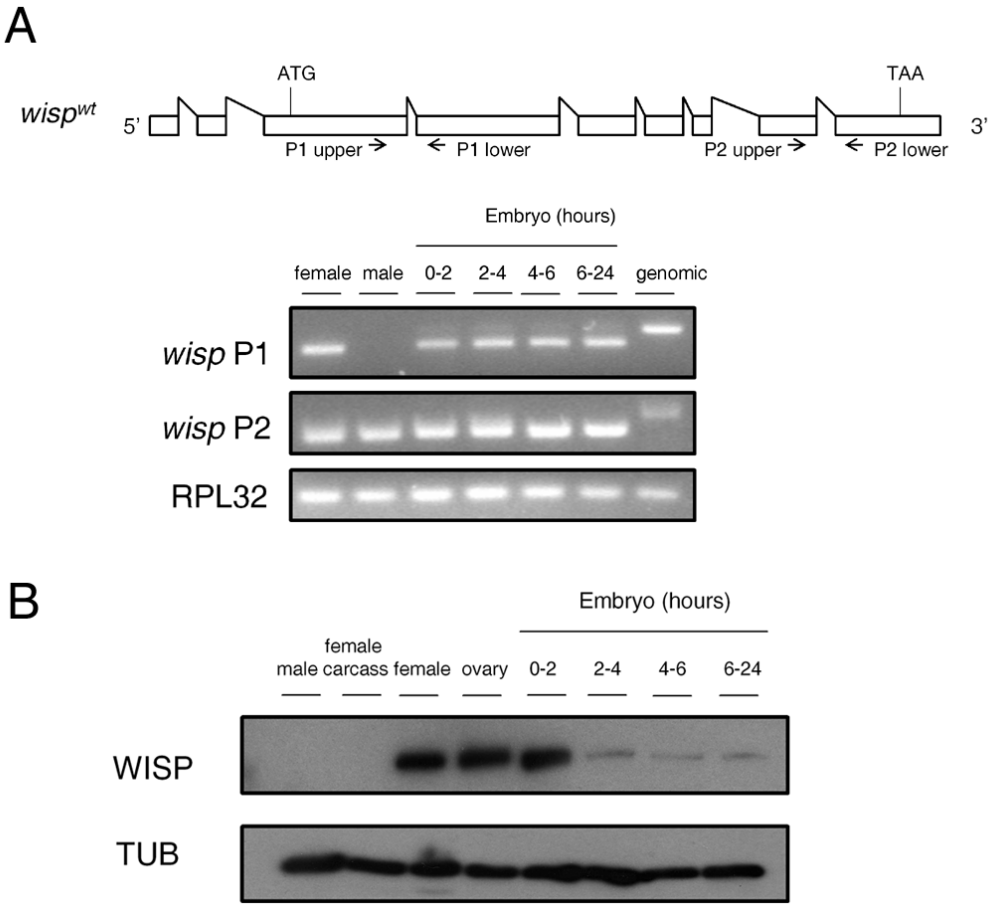
embryogenesis with the primer set P2 which amplifies a region near the 3' end of the coding sequence (Figure 2.3A). The primer set P1, located towards the 5' end of the gene, amplifies a PCR product of the predicted size in cDNAs made from female adult flies and all stages of deposited eggs or embryos, but does not amplify any PCR product in cDNA made from male flies. These results indicate presence of *wisp* mRNA in females and in eggs/embryos, and suggest that males lack the long form of this RNA predicted by bioinformatics. Since I focus here on the roles of WISP in the female germline, I did not investigate the structure of the male transcript further.

To assay for the presence of WISP protein, I performed Western blot analysis with my WISP antibody. I find that high levels of WISP protein are present in adult female flies, ovarian oocytes and 0-2 hr embryos. WISP protein is not detected in adult male flies or non-ovarian female tissues (Figure 2.3B). Low levels of WISP protein are also detected in late stage embryos.

The second GLD-2 encoded by CG5732 is expressed in the testis: The Drosophila genome contains a second GLD-2 family member (CG5732). To determine the expression pattern of DmGLD-2, I extracted RNA from male or female flies and performed RT-PCR using gene specific primers. Transcripts are detected only in cDNA made from RNA extracted from adult male flies but not in females (Figure 2.4A). A recent study reported that DmGLD-2 was expressed in the Drosophila nervous system (Kwak et al., 2008). This expression could be below the detection limit of my RT-PCR assay.

To assay for the presence of DmGLD-2 protein, I generated an antibody against DmGLD-2 (see Materials and Methods) and performed Western blot analysis. I find high levels of DmGLD-2 protein in testes but not detectable in ovaries. The reciprocal pattern is seen for WISP protein; it is detected only in female gonads but

Figure 2.3 Presence of *wisp* transcripts and proteins. (A) Total RNAs were extracted from 3- to 4- day-old wild type adult female and male flies or laid embryos of the indicated ages. mRNA was reverse transcribed into cDNA for PCR analysis. RPL32 primers were used as positive control. Two different sets of *wisp* primers shown in the context of the *wisp* mRNA as predicted in FlyBase (www.flybase.org) were used to detect *wisp* transcripts. A PCR product for *wisp* P1 is present in all samples examined except male cDNA. PCR products for both *wisp* P2 and RPL32 are present in all samples. (B) Ovaries were dissected from 3- to 4- day-old virgin females and the females' remaining somatic tissues were used as the female carcass sample. Total protein extracts from these two samples, wild type adult flies and laid embryos of the indicated ages, were separated by SDS-PAGE, blotted and probed for anti-WISP and anti-α-tubulin (as a loading control).


A
wisp^wt
ATG
TAA
5'
3'
P1 upper
P1 lower
P2 upper
P2 lower
Embryo (hours)
female
male
0-2
2-4
4-6
6-24
genomic
wisp P1
wisp P2
RPL32
B
Embryo (hours)
female
male carcass
female
ovary
0-2
2-4
4-6
6-24
WISP
TUB

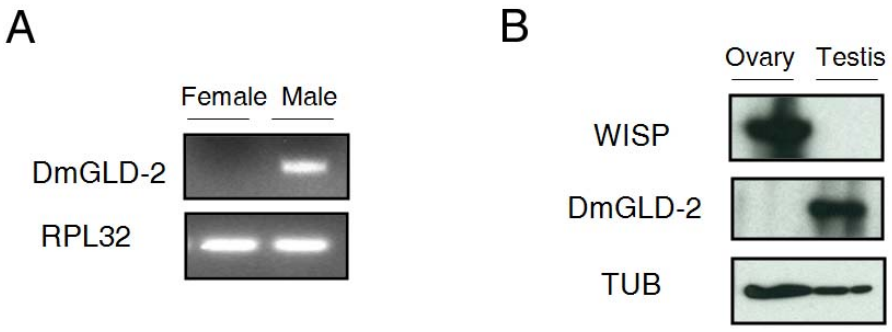


Figure 2.4 Presence of DmGld-2 transcripts and proteins. (A) Total RNAs were extracted from 3- to 4- day-old wild type adult female and male. mRNA was reverse transcribed into cDNA for PCR analysis. RPL32 primers were used as positive control. Primers specific for DmGld-2 coding sequence as predicted in FlyBase (www.flybase.org) were used to detect DmGld-2 transcripts. DmGld-2 PCR product is detected only in male cDNA. PCR product of RPL32 is present in both samples. (B) Ovaries were dissected from 3- to 4- day-old virgin females and testes were dissected from 3- to 4- day-old old males. Total protein extracts from these two samples were separated by SDS-PAGE, blotted and probed for anti-WISP, anti-DmGLD-2 and anti-α-tubulin (as a loading control).

not in male gonads (Figure 2.4B). These results suggest that the two GLD-2 proteins are expressed in a sex-dependent manner and might have sex-specific functions.

Consistent with this, further analysis showed that WISP is required for female germ cell development; *wisp* mutations cause multiple defects in oocyte development (see Chapter 3). Studies by C. Sartain show that DmGLD-2 is required for male germ cell development, specifically for elongation and individualization stages of spermatogenesis (Sartain, Cui and Wolfner, in prep).

Drosophila GLD-2 proteins can interact with Bicaudal-C: Sequence comparisons suggest that both WISP and DmGLD-2's primary amino acid sequences, like those of other GLD-2 proteins (Kwak et al., 2004; Kwak and Wickens, 2007), do not contain any predicted RNA recognition motifs. In *C. elegans*, binding of GLD-2 to its target mRNAs is instead mediated by an RNA binding protein GLD-3 (Wang et al., 2002). In Xenopus, xGLD-2 is found in a cytoplasmic polyadenylation complex in which CPEB and CPSF are also present (Barnard et al., 2004). Drosophila has homologs of some of these proteins, and the Drosophila CPEB, encoded by the *orb* gene, has been shown to interact with Bicaudal-C (Bic-C), the Drosophila homolog of GLD-3 (Castagnetti and Ephrussi, 2003). Bic-C, which is present in oocytes and early embryos like WISP, is required for anterior-posterior patterning during oogenesis (Mahone et al., 1995; Saffman et al., 1998). In the Drosophila female germline, Bic-C was reported to regulate the expression of specific mRNAs by participating in control of their poly(A) tail length (Chicoine et al., 2007).

Using the yeast two-hybrid assay, I found that both WISP and DmGLD-2 can interact with Bic-C (Table 2.1). Either GLD-2 family member fused in frame to an activation domain (AD) can interact with Bic-C fused in frame to a binding domain (BD), but they do not interact with the BD alone (that is, with an empty vector). I only observed interaction between GLD-2s and Bic-C in one orientation. Both WISP-BD

Table 2.1 Interaction between the two Drosophila GLD-2 proteins and Bic-C in yeast two-hybrid assay.

AD: activation domain

BD: DNA binding domain

+: growth on -Trp -Leu -His and -Trp -Leu -His -Ade media

-: no growth on -Trp -Leu -His or -Trp -Leu -His -Ade media

	AD	AD-WISP	AD-DmGLD-2
BD	-	-	-
BD-Bic-C	-	+	+

and DmGLD-2-BD fusions were not detected on Western blots, suggesting that those BD fusion proteins were either not expressed or not stable in yeast (data not shown). The two-hybrid interaction that I observed between GLD-2 proteins and Bic-C suggests that WISP and DmGLD-2 in Drosophila might both function, analogous to the GLD-2 proteins in other animals, by forming a protein complex with GLD-3 RNA binding proteins.

In summary, I have shown that the two members of the GLD-2 cytoplasmic PAP family in the *Drosophila melanogaster* genome are expressed in the gonads, and that their expression patterns are sex-dependent. One GLD-2, encoded by the *wisp* gene, is specifically expressed in the female germ-line cells. The other GLD-2, encoded by the *gld-2* (CG5732) gene, is expressed in the testis of the male flies. Both GLD-2 proteins can associate with Bic-C, the Drosophila GLD-3 homolog.

CHAPTER 3

WISPY, THE FEMALE SPECIFIC GLD-2, IS REQUIRED DURING OOGENESIS AND EGG ACTIVATION[2]

3.1 Introduction

Mature oocytes of most animals are arrested at a species-specific stage of meiosis until certain events occur that convert the oocyte to a state that can undergo embryogenesis. Prior to these events, the mature oocyte is developmentally "poised": it contains maternal mRNAs and other molecules that are required for embryogenesis. The process that transitions the oocyte to become competent for further development is called egg activation. In vertebrates and marine invertebrates, egg activation is triggered by fertilization. Entry of the sperm causes an increase of free calcium in the egg cytoplasm, which triggers the completion of meiosis (Ducibella et al., 2006; Parrington et al., 2007). In Drosophila, egg activation occurs independently of fertilization and appears to be triggered by mechanical stress experienced by the egg when it passes through the female reproductive tract during ovulation (Heifetz et al., 2001). A calcium-dependent signaling pathway is involved in activation of Drosophila eggs (Horner et al., 2006; Takeo et al., 2006), and many of the events of Drosophila egg activation downstream of the initial trigger appear analogous to those in other animals.

Activation events include modifications in egg coverings, resumption and completion of meiosis, degradation of some maternal mRNAs, a decrease in mitogen-activated protein kinase (MAPK) activity, and changes in the cytoskeleton (Heifetz et

[2] Portions of this chapter were published in Cui et al. (Cui et al., 2008), and are reprinted with permission. Figures 3.3B, 3.7A and 3.7B are contributed by K. Sackton.

al., 2001). An additional crucial aspect of egg activation is the polyadenylation of some maternal transcripts that initiates their translation. In Drosophila, transcripts subject to regulation by polyadenylation during egg activation include the *bicoid*, *Toll*, and *torso* mRNAs. These mRNAs encode proteins that are involved in defining embryo polarity. The protein products of these mRNAs are first detected only after egg activation is triggered (Casanova and Struhl, 1989; Driever and Nusslein-Volhard, 1988; Hashimoto et al., 1988). Initiation of the translation of these mRNAs correlates with an increase in the length of their poly(A) tails (Salles et al., 1994).

Cytoplasmic polyadenylation is a key regulatory mechanism that controls the function of maternal mRNAs during the early development of Xenopus oocytes (Kadyk and Kimble, 1998; Mendez and Richter, 2001). In Xenopus oocytes, the cytoplasmic polyadenylation element binding protein (CPEB) recognizes the cytoplasmic polyadenylation element (CPE) in the 3' UTR of the mRNA (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). CPEB associates with the cleavage and polyadenylation specificity factor (CPSF), which recognizes another 3' UTR element, AAUAAA (Dickson et al., 1999; Dickson et al., 2001; Mendez et al., 2000b). GLD-2 is thus recruited to mRNA through interaction with CPEB and CPSF (Barnard et al., 2004). Several mRNAs required for oocyte maturation in Xenopus, such as those encoding Cyclin B and Mos, are controlled by this machinery (Barkoff et al., 2000; Groisman et al., 2000; Mendez et al., 2000a).

I have shown that *wisp*, one of the two Drosophila GLD-2 proteins, is expressed specifically in the female germline cells. *wisp* maternal-effect mutations were previously reported to cause defects in *bicoid* mRNA localization and in microtubule-based events of female meiosis, leading to very early developmental arrest (Brent et al., 2000). Early embryos of *wisp* mutant females were also known to fail to destabilize maternal transcripts, a phenotype that suggested a defect in egg

activation (Tadros et al., 2003). To further dissect *wisp*'s possible role in oogenesis and egg activation, I assessed the ability of *wisp* mutants to undertake various events of late oogenesis and egg activation. I find that *wisp* mutant embryos are defective in the addition of poly(A) to several maternal mRNAs during egg activation. Embryos from *wisp* mutant mothers also fail to initiate mitosis after fertilization. This problem appears to result in part from abnormalities that occur during the completion of female meiosis in the absence of *wisp* function: the acentriolar microtubule organizing center between meiosis II spindles is reduced in size, pronuclei fail to appose and the polar body rosette does not form. WISP function is also required in oocytes for the normal, regulated polyadenylation of *dmos* mRNA and, potentially through this action, for phosphorylation (activation) of MAPKs in mature oocytes prior to egg activation.

3.2 Materials and Methods

Drosophila stocks: Oregon-R was used as the wild type stock. $wisp^{41}$/FM6, $wisp^{248}$/FM6 and $wisp^{249}$/FM6 (Tadros et al., 2003) were kindly provided by W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). $wisp^{12\text{-}3147}$/FM0, described by (Brent et al., 2000), was obtained from the Bloomington Drosophila Stock Center (Indiana). sra^{687}/FM6 and *Df(3R)sbd45,* mwh^1 e^1 /TM6 were previously described by (Horner et al., 2006).

Embryo collection, fixation and staining: Laid eggs were collected as previously described (Horner et al., 2006). Three to 4-day-old virgin females were mated either to wild-type Oregon-R males to produce fertilized eggs (embryos) or to spermless males [the sons of tud^1 *bw sp* mothers (Boswell and Mahowald, 1985)] to generate unfertilized activated eggs.

Fertilized and unfertilized eggs were collected on grape juice plates for the desired period of time and washed off the plates with egg wash buffer (0.4% NaCl, 0.2%

Triton X-100). Hereafter, "0- to 1-hr embryos" will refer to fertilized eggs collected 0- to 1-hr post egg deposition, and other collection lengths will be described in similar fashion. "Eggs" will refer to eggs laid by females mated to wild-type males, which may be fertilized or unfertilized, and "unfertilized eggs" will refer to eggs laid by females mated to spermless males (described above), unless otherwise indicated. For immunofluorescence analysis, collected eggs were dechorionated in 50% bleach for 2 min, rinsed thoroughly with water, then permeabilized with heptane and fixed immediately in cold methanol.

Fixed eggs were washed in 1 X PBST (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Triton X-100) and incubated at 4° overnight with a 1:200 dilution of a mouse anti-α-tubulin antibody (Sigma, St. Louis, MO) in 1 X PBST. RNaseA (Roche Applied Science, Indianapolis, IN) was added to a final concentration of 5 μg/ml. Secondary antibody [Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:200] was then added for 2 hr at room temperature. Propidium iodide (Invitrogen) was added at a concentration of 10 μg/ml to stain DNA. Samples were either mounted in 75% glycerol containing 940 mM *n*-propyl gallate or washed with methanol and mounted in benzyl benzoate:benzyl alcohol (2:1).

Staining in fertilized and unfertilized eggs was analyzed using confocal microscopy [Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope (Leica Microsystems, Germany)]. Leica software was used to collect images and, where appropriate, to project multiple optical sections into a single image and to overlay images.

Western blot analysis: Samples from ovarian oocytes and 0- to 15-min or 0- to 2-hr embryos were prepared for Western blotting. Anti-SMG antibody, a kind gift from W. Tadros and H. Lipshitz, was diluted 1:10,000 (Tadros et al., 2007). Anti-α-

tubulin antibody (Sigma, catalog #T5168) was diluted 1:10,000. Anti-MAPK antibodies were used as previously described (Sackton et al., 2007).

Poly(A) tail (PAT) assay: Oocytes were dissected from 3-4 day old virgin control and virgin *wisp* mutant females. 0-1 hr embryos were collected as described above and aged at room temperature for 1 or 2 hours to get 1-2 or 2-3 hr embryos, respectively. Total RNA was extracted using TRIzol (Invitrogen), and 1μg total RNA from each sample was used for poly(A) tail (PAT) assay. PCR-based PAT assays were performed as described in (Salles and Strickland, 1995). Briefly, total RNA from each sample was incubated in the presence of 20 ng 5'-phosphorylated oligonucleotide $p(dT)_{18}$ to saturate the poly(A) tails of the mRNAs. The $p(dT)_{18}$ was then ligated together in the presence of 10 units (Weiss) of T4 DNA ligase (Fermentas, Glen Burnie, MD) to generate a complementary copy of the poly(A) tail. Two hundred nanograms of oligo$(dT)_{12}$-anchor was added and ligated to the 5'-end of the poly(T) strand. The mRNAs were then reverse transcribed (as described above) to synthesize the PAT cDNAs. PCR was performed on the PAT cDNAs using a gene-specific primer and the oligo$(dT)_{12}$-anchor to test the length of the poly(A) tail of a specific mRNA. Sequences of primers specific for *bicoid*, *Toll*, and *torso* and the sequence of the oligo$(dT)_{12}$-anchor were described by (Salles et al., 1994). The sequence of the primer specific for *dmos* is 5'-GCTGAAGCATGAGCTGGAATTC. PCR products from the PAT assays were run on 2% agarose gels or 8% acrylamide gels. Gels were stained with 0.5 μg/ml ethidium bromide.

3.3 Results and Discussion

***bicoid* poly(A) tail length does not increase in *wisp* mutant embryos:** Given that the WISP protein is a GLD-2 homolog, I wished to determine whether it is involved in modulating the length of the poly(A) tail of maternal mRNAs during egg

activation. I focused first on *bicoid (bcd)*, whose product is essential for anterior structure formation in Drosophila embryos (Driever and Nusslein-Volhard, 1988). Maternal *bcd* mRNA remains untranslated until it receives an extended poly(A) tail during egg activation (Salles et al., 1994; Surdej and Jacobs-Lorena, 1998).

I used a PCR-based poly(A) test (PAT) (Salles and Strickland, 1995) to measure the length of the poly(A) tail on *bcd* mRNA in ooctyes and laid (that is, activated) embryos. I obtained these oocytes and embryos from mated "*wisp* females" that are either homozygous for a *wisp* allele or carry one *wisp* mutant allele over a deficiency of the *wisp* locus, *Df(1)RA47*. Heterozygous sibling females with one copy of a *wisp* mutant allele and one copy of a balancer chromosome carrying a wild-type $wisp^{+}$ allele were used as controls. In embryos from $wisp^{41}$/+ and $wisp^{248}$/+ control females, *bcd* poly(A) tail length, which is ~70 nt in oocytes, increases in 0- to 1-hr embryos (after activation), reaching a peak length of ~140 nt (Figure 3.1). These observations are consistent with previous reports for the magnitude of the change in *bcd* poly(A) tail length during egg activation (Salles et al., 1994). However in 0- to1-hr embryos from $wisp^{41}/Df(1)RA47$ and $wisp^{248}/wisp^{248}$ females, the poly(A) tail of *bcd* mRNA does not show this increase in length. Rather, there is a small (~40 nt) decrease of the length of the *bcd* poly(A) tail in embryos from *wisp* females (Figure 3.1). Thus, WISP protein is required for the extension of the *bcd* poly(A) tail upon activation. A short poly(A) tail could affect translation of the BCD protein. Although (Tadros et al., 2003) reported that BCD is translated in *wisp* mutants, a shorter poly(A) tail could affect translation efficiency, leading to a reduced level of BCD protein in *wisp* mutants. Unfortunately, I was unable to quantify BCD protein levels because no suitably specific antibody is available. Two other maternal mRNAs, *Toll* and *torso*, that are also polyadenylated upon activation (Salles et al., 1994) similarly fail to acquire extended poly(A) tails in *wisp* mutants (Figure 3.2).

Figure 3.1 Failure of *bicoid* mRNA polyadenylation in embryos from *wisp* mothers. Total RNA was extracted from ovarian oocytes or different stages of embryos from females of genotypes noted in the figure. PAT assays were performed to analyze the length of the *bcd* poly(A) tail. Tail lengths were estimated based on molecular size markers. *bcd* poly(A) tail length increased in control embryos after egg activation (left). Polyadenylation of *bcd* mRNA does not occur in *wisp* mutant embryos (right). Rather there is a ~40 nt decrease of the length of *bcd* poly(A) tail. PAT assays were performed on two independent sets of $wisp^{41}$ or control samples and one set of $wisp^{248}$ and control samples.

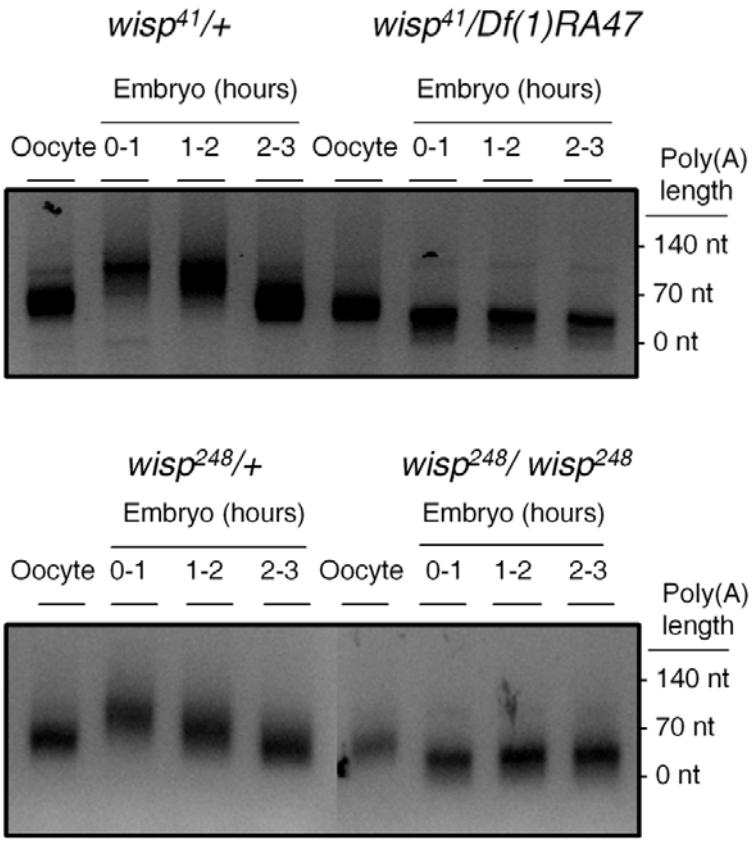
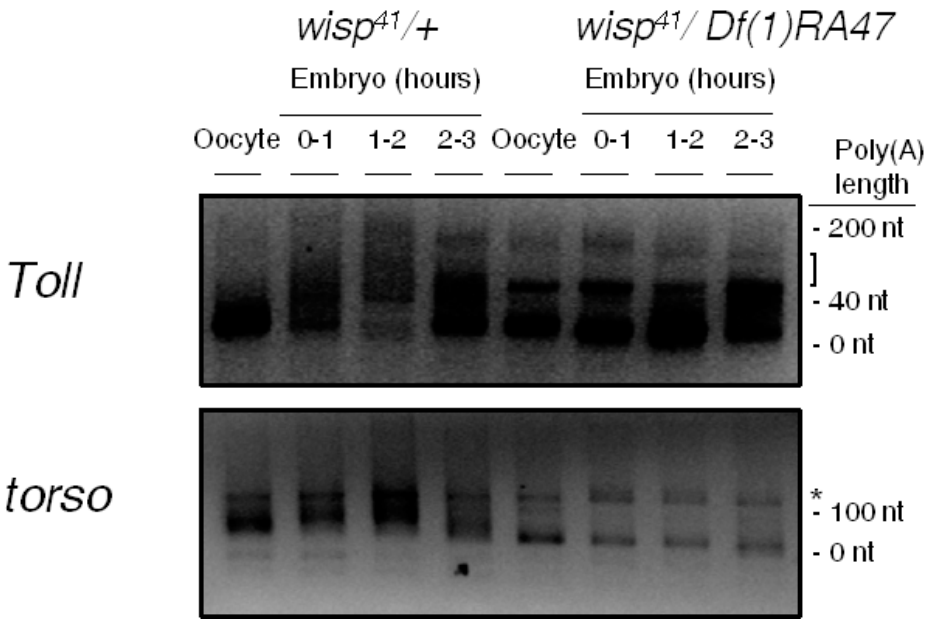

wisp41/+
wisp41/Df(1)RA47
Embryo (hours)
Embryo (hours)
Oocyte
0-1
1-2
2-3
Oocyte
0-1
1-2
2-3
Poly(A) length
140 nt
70 nt
0 nt
wisp248/+
wisp248/ wisp248
Embryo (hours)
Embryo (hours)
Oocyte
0-1
1-2
2-3
Oocyte
0-1
1-2
2-3
Poly(A) length
140 nt
70 nt
0 nt

Figure 3.2 Failure of *Toll* and *torso* mRNA polyadenylation in embryos from *wisp* mothers. Total RNA was extracted from ovarian oocytes or different stages of embryos from females of genotypes noted in the figure. PAT assays were performed to analyze the lengths of the poly(A) tail. Tail lengths were estimated based on molecular size markers. In controls, the poly(A) tail lengths of *Toll* mRNA (top left) and *torso* mRNA (bottom left) increase after egg activation (0- to 1-hr embryos compared to unactivated ovarian oocytes). The extension of poly(A) tails of *Toll* and *torso* mRNAs continues until 1- to2-hr and 0- to 1- hr after egg deposition, respectively. However the poly(A) tail length of *Toll* mRNA (top right) and *torso* mRNA (bottom right) do not increase in *wisp*-deficient embryos. Levels of *Toll* mRNA detected in control embryos decrease by 1- to 2-hr, and then increase. We believe that the increase seen at 2-3 hr could reflect zygotic transcription. Asterisk shows a secondary PCR product amplified by the *torso* primer. Bracket shows bands reproducibly detected in both oocytes and embryos of the *wisp/Df* genotype but not in controls, which are probably nonspecific PCR products amplified by the *Toll* primer.


wisp41/+
wisp41/ Df(1)RA47
Embryo (hours)
Embryo (hours)
Oocyte
0-1
1-2
2-3
Oocyte
0-1
1-2
2-3
Poly(A) length
Toll
- 200 nt
- 40 nt
- 0 nt
torso
*
- 100 nt
- 0 nt

Cytoplasmic polyadenylation of maternal mRNAs is a conserved mechanism that regulates translation during both oogenesis and early embryo development. Previous studies of GLD-2 family PAPs revealed that GLD-2 plays an important role in the meiosis/mitosis decision in *C. elegans* (Kadyk and Kimble, 1998) and in oocyte maturation in both Xenopus (Barnard et al., 2004) and mouse (Nakanishi et al., 2006). However, those studies focused on cytoplasmic polyadenylation events during oogenesis; how cytoplasmic elongation of poly(A) tails is regulated following egg activation remains, in contrast, poorly understood. Mature oocytes contain a remarkable array of maternal mRNAs, representing 20-45% of the genes in the mouse genome and 55% of the genes in Drosophila (Evsikov et al., 2006; Tadros et al., 2007; Wang et al., 2004). The translation of these maternal mRNAs is a hallmark of egg activation, because 2D gel electrophoresis in mouse (Xu et al., 1994) and sea urchin (Roux et al., 2006) shows a tremendous spike in new protein synthesis within the egg immediately after fertilization. Initiation of the translation of some mRNAs correlates with an increase in the length of their poly(A) tails (Salles et al., 1994). I find that *wisp*, which encodes a Drosophila homolog of GLD-2 proteins, is essential for regulating polyadenylation of *bicoid*, *Toll* and *torso* transcripts upon activation. There might be other maternal mRNAs that are regulated by their poly(A) tail lengths in a *wisp*-dependent manner during Drosophila egg activation, and it will be interesting in the future to identify these mRNAs.

It will also be interesting to know how the *wisp*-dependent cytoplasmic polyadenylation machinery is turned on during egg activation. Calcium signaling pathways are major transducers of the egg activation trigger in many systems (Ducibella et al., 2006; Parrington et al., 2007), so perhaps cytoplasmic polyadenylation is regulated by a calcium signal. A recent study shows calcium involvement in egg activation in Drosophila (HORNER and WOLFNER 2008a). In

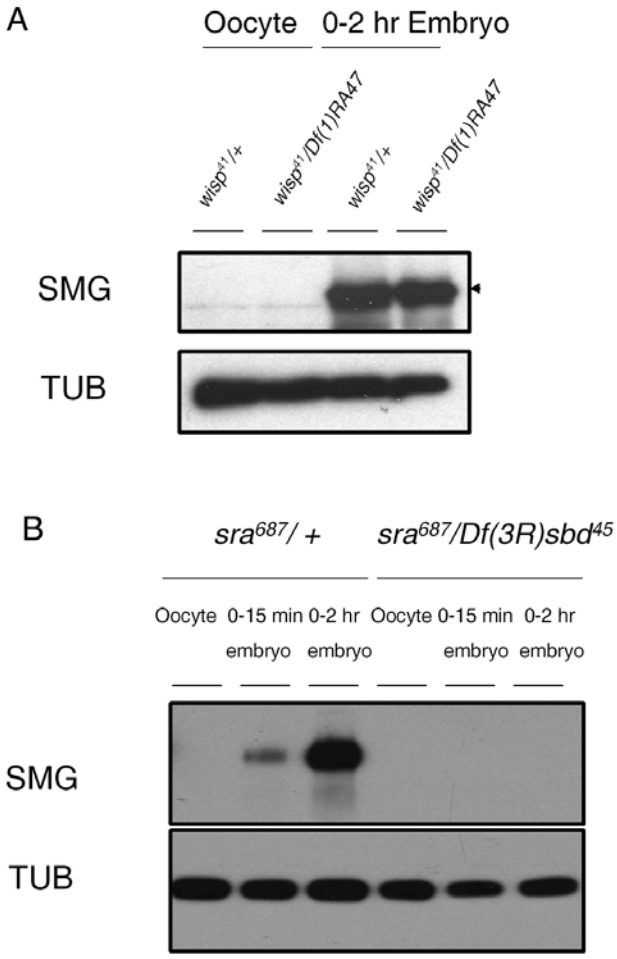
addition, studies of the *sarah (sra)* gene (Horner et al., 2006; Takeo et al., 2006), which encodes the Drosophila calcipressin, indicate that proper regulation of the calcium-dependent phosphatase calcineurin by Sra is required for egg activation. Horner et al., (Horner et al., 2006) showed that *bcd* mRNA poly(A) tails do not extend in early embryos from *sra* mutant mothers. This latter result suggests that the calcium signaling pathway might be upstream of polyadenylation during Drosophila egg activation. Consistent with the idea that cytoplasmic polyadenylation could be calcium-regulated, a study of hippocampal neurons in mouse showed that CaMKII can phosphorylate a regulatory site on CPEB to activate cytoplasmic polyadenylation (Atkins et al., 2004). In a model where calcium is upstream of cytoplasmic polyadenylation upon egg activation, calcium could act upstream of WISP, or upstream of other possible targets such as those GLD-2 associated factors in other organisms whose homologs are also found in Drosophila, such as Orb and Bic-C. Since cytoplasmic polyadenylation can be affected by the rate of deadenylation, and deadenylation is also turned on upon activation (Tadros et al., 2007; Tadros et al., 2003; Tadros and Lipshitz, 2005), the calcium signaling pathway could also potentially act on targets in the deadenylation pathway, such as Smaug (Semotok et al., 2005; Tadros et al., 2007), during activation.

WISP is not required for SMG translation: In Drosophila, egg activation triggers destabilization of over 1600 maternal mRNAs (Tadros et al., 2007). This destabilization has been hypothesized to eliminate maternal transcripts so that zygotic transcription can control later embryogenesis. Smaug (SMG) is the major regulator of this mRNA destabilizing activity. SMG protein is not present in mature oocytes but is present in embryos (Dahanukar et al., 1999; Smibert et al., 1996). *smg* mRNA has been reported to be polyadenylated upon egg activation, correlating with, but not absolutely required for, its translation (Tadros et al., 2007). Since WISP is also

required for maternal transcript destabilization during egg activation (Tadros et al., 2003), I tested whether SMG protein is translated in embryos in the absence of *wisp* function. On Western blots, I find that SMG protein is detected in 0- to 2-hr embryos from *wisp*^{41} mutant mothers at levels comparable to those in control embryos (Figure 3.3A). This result indicates that the translation of SMG does not require *wisp* function. In the future it would be interesting to test if *wisp* affected the *smg* transcript's poly(A) tail length.

SMG triggers transcript degradation by recruiting the CCR4/POP2/NOT deadenylase complex to specific maternal transcripts to destabilize these mRNAs (Semotok et al., 2005; Tadros et al., 2007). My finding that SMG is translated in embryos from *wisp* mutant mothers in conjunction with my previous observation of a small (~40 nt) decrease in the length of the *bcd* poly(A) tail in *wisp* mutant embryos (Figure 3.1) suggests that the shortening of the *bcd* poly(A) tail in *wisp*-deficient embryos might be due to a SMG-dependent increase in deadenylase activity upon activation. Upon egg activation, SMG-dependent deadenylation of the *bcd* transcript would normally be counteracted by polyadenylation, but this deadenylation could become detectable when polyadenylation is disrupted, as in the *wisp* mutant. Given that polyadenylation of *bcd* transcript is affected in embryos from *sra* mutants (HORNER *et al.* 2006), my co-author and I also considered the role of SMG-dependent deadenylation in *sra* mutants. Horner et al., (2006) showed that *bcd* mRNA's poly(A) tail is not extended in embryos laid by *sra* mutant mothers, but in contrast to my observation for *wisp* mutants, these investigators saw no decrease in the length of the *bcd* mRNA poly(A) tail in the absence of *sra*. If the deadenylation of *bcd* mRNA in *wisp* mutants is caused by the activation of SMG protein, then the lack of *bcd* transcript poly(A) decrease in *sra* embryos might suggest that *sra* eggs and embryos do not activate SMG translation. Consistent with this prediction, my co-author K.

Figure 3.3 SMG protein is translated in *wisp* mutant embryos, but not in *sra* embryos. (A) Total protein extracts of ovarian oocytes (left) and laid 0- to 2-hr embryos (right) from $wisp^{41}/Df(1)RA47$ or $wisp^{41}/+$ females were separated by SDS-PAGE, blotted, and probed for Smaug (SMG) and α-tubulin (Tub; loading control). SMG protein is translated normally in laid embryos from *wisp* mutant females; there is no statistically significant difference between control and mutant embryos in the amount of SMG signal intensity, normalized to Tub signal intensity, in 5 independent replicates (Matched Pairs T-test: t-ratio = -0.205, 4 degrees of freedom, p=0.848) . (B) Total protein extracts of oocytes, 0- to 15-min embryos and 0- to 2-hr embryos from $Sra^{687}/+$ or $Sra^{687}/Df(3R)sbd^{45}$ females were isolated, blotted, and probed for SMG and α-tubulin (Tub; loading control). SMG protein is not detected in embryos from *sra* mutant females.


A
Oocyte
0-2 hr Embryo
wisp41/+
wisp41/Df(1)RA47
wisp41/+
wisp41/Df(1)RA47
SMG
TUB
B
sra687/ +
sra687/Df(3R)sbd45
Oocyte
0-15 min embryo
0-2 hr embryo
Oocyte
0-15 min embryo
0-2 hr embryo
SMG
TUB

Sackton find that embryos from *sra* mutant females lack detectable SMG (Figure 3.3B).

WISP and SMG are both required for maternal transcript destabilization during egg activation (Tadros et al., 2003). The function of WISP in regulating maternal transcript destabilization cannot be mediated through regulation of SMG translation, because SMG is still translated in *wisp*-deficient early embryos. Translation of SMG protein upon egg activation is instead known to be activated by the PAN GU (PNG) kinase complex (Tadros et al., 2007), and PNG also plays a role in promoting the polyadenylation of *smg* mRNA (Tadros et al., 2007) and *cyclin B* mRNA (Vardy and Orr-Weaver, 2007). Future studies are needed to determine whether WISP functions in mRNA destabilization downstream of PNG or instead functions in parallel to the PNG-SMG pathway.

Embryos from *wisp* mutant females arrest subsequent to the completion of female meiosis but prior to the apposition of female and male pronuclei: Because *wisp* mutants fail to carry out two aspects of egg activation [polyadenylation of maternal RNAs (this study) and maternal mRNA destabilization (Tadros et al., 2003)], I asked whether other aspects of egg activation are affected in *wisp* mutants. Modifications to the egg coverings are an essential part of egg activation in many species. The vitelline membrane, one of Drosophila's egg coverings, is permeable to small molecules before activation, but after activation vitelline membrane proteins become crosslinked to render the activated egg impermeable to small molecules (Heifetz et al., 2001; LeMosy and Hashimoto, 2000). Activated eggs are consequently impermeable to bleach and remain resistant to lysis after a 2-min incubation in 50% bleach, while ~100% of eggs that have not yet been activated are lysed by this treatment (Mahowald et al., 1983). Zero- to 1-hr eggs laid by either *wisp/+* (control) or *wisp/wisp* females were collected and assayed for bleach resistance. In one

representative experiment, 90.5% of *wisp*^{41}/+ (N=412) eggs and 92.2% of *wisp*^{248}/+ (N=516) eggs were resistant to 50% bleach after a 2-min incubation, while 60.7% of the eggs (N=384) laid by *wisp*^{41}/*wisp*^{41} (a presumptive null mutation) females and 68.1% of the eggs (N=426) laid by *wisp*^{248}/*wisp*^{248} females were resistant to bleach, suggesting that vitelline membrane crosslinking occurs in *wisp* mutants, but might be incomplete or delayed relative to wild type ($p<0.0001$). While the exact percentage values vary among other repetitions of this experiment, the difference between controls and *wisp* mutants remains in the same direction and always statistically significant.

Another aspect of egg activation is the resumption and completion of female meiosis. (Brent et al., 2000) reported that embryos laid by *wisp*$^{12\text{-}3147}$, *wisp*$^{11\text{-}600}$ and *wisp*$^{14\text{-}1299}$ females arrest after an abnormal meiosis. Since *wisp*$^{12\text{-}3147}$ is a missense (or double missense) mutation and the molecular lesions in the other two alleles tested by (Brent et al., 2000) are unknown, I was uncertain if they reflect the null phenotype. I therefore chose to analyze two other alleles, *wisp*^{41} and *wisp*^{248}, because the *wisp*^{41} allele has a nonsense mutation that removes the majority of the protein including the entire conserved PAP domain and thus is likely to be the strongest allele available. I further analyzed the progression of cell cycle phenotype due to *wisp*^{41} and *wisp*^{248}, and determined that both meet the genetic definition of null alleles (Table 3.1). Both *wisp*^{41} and *wisp*^{248} females are viable and capable of laying eggs, but these eggs never hatch. I examined the phenotypes of these arrested eggs using immunofluorescence microscopy. Control and *wisp* females were mated to wild type males, and the resultant eggs were collected. They were then fixed and stained with an antibody against α-tubulin to visualize the spindle, and with propidium iodide to reveal the chromosomes. Eggs from hemizygous (*wisp*^{41}*/Df(1)RA47* or *wisp*^{248}*/Df(1)RA47*) females show the same phenotype as those from homozygous (*wisp*^{41}/*wisp*^{41} or

Table 3.1 Distribution of developmental stages of 0-2 hr old embryos. Embryos collected from females of the indicated genotype were fixed and stained with propidium iodide and anti-α-tubulin antibody. Stages were determined based on spindle and chromosome configurations.

Genotype	Meiosis	Mitosis	Arrested
wisp^{41}/FM6	2/39	37/39	0/39
wisp^{41}/ wisp^{41}	0/20	0/20	20/20
wisp^{41}/Df(1)RA47	1/24	0/24	23/24
wisp^{248}/FM6	3/33	30/33	0/33
wisp^{248}/ wisp^{248}	0/31	0/31	31/31
wisp^{248}/Df(1)RA47	0/27	0/27	27/27

wisp^{248}/*wisp*^{248}) females. Since the *wisp*^{41} allele has a nonsense mutation that removes the majority of the protein including the entire conserved PAP domain and meets the genetic definition of a null, I focused further phenotypic analysis on this allele.

In wild type embryos, female meiosis completes upon egg activation and four haploid meiotic products form (Foe et al., 1993). If the egg is fertilized, the sperm nucleus remodels (in part by exchanging protamines for histones) and becomes the male pronucleus. One of the four female meiotic products migrates toward, and apposes with, the male pronucleus, and then the two apposed pronuclei together enter the first mitotic division. The other three female meiotic products stay at the cortex of the embryo and form a polar body rosette. I examined whether or not *wisp* eggs can be fertilized, and whether meiosis and pronuclear behaviors are normal. Asters can be observed using α-tubulin antibody and the sperm tail can be observed using an antibody against the sperm tail in embryos from *wisp*^{41} mutant females (Figure 3.4) as was also stated for *wisp*$^{12\text{-}3147}$ mutants (Brent et al., 2000). Thus, the lack of development of *wisp* embryos is not due to lack of fertilization.

As expected, the majority of 0- to 1-hr fertilized eggs (embryos) laid by *wisp*^{41}/+ heterozygous females are undergoing embryonic mitotic divisions (Figure 3.5A). In contrast, no embryos from *wisp* homozygous mutant females enter a normal zygotic mitosis. Instead they are arrested at an aberrant metaphase-like stage with very few (usually five or fewer) spindles (Figure 3.5B). The chromosomes associated with the spindle structures are condensed and appear to have entered a division cycle, since their chromatin can be stained by an antibody against the mitosis marker phospho-H3 (data not shown). The spindles in these arrested embryos are clearly different from mitotic spindles, since they are in general not associated with microtubule organizing centers (MTOC). However, one spindle deep inside the embryo, with two aster structures, can be observed in some of these fertilized eggs (Figure 3.5B inset).

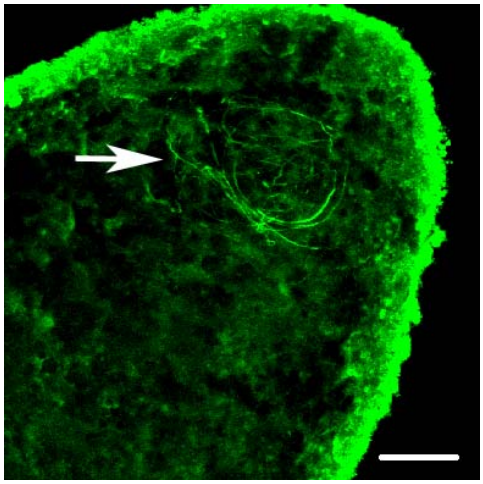
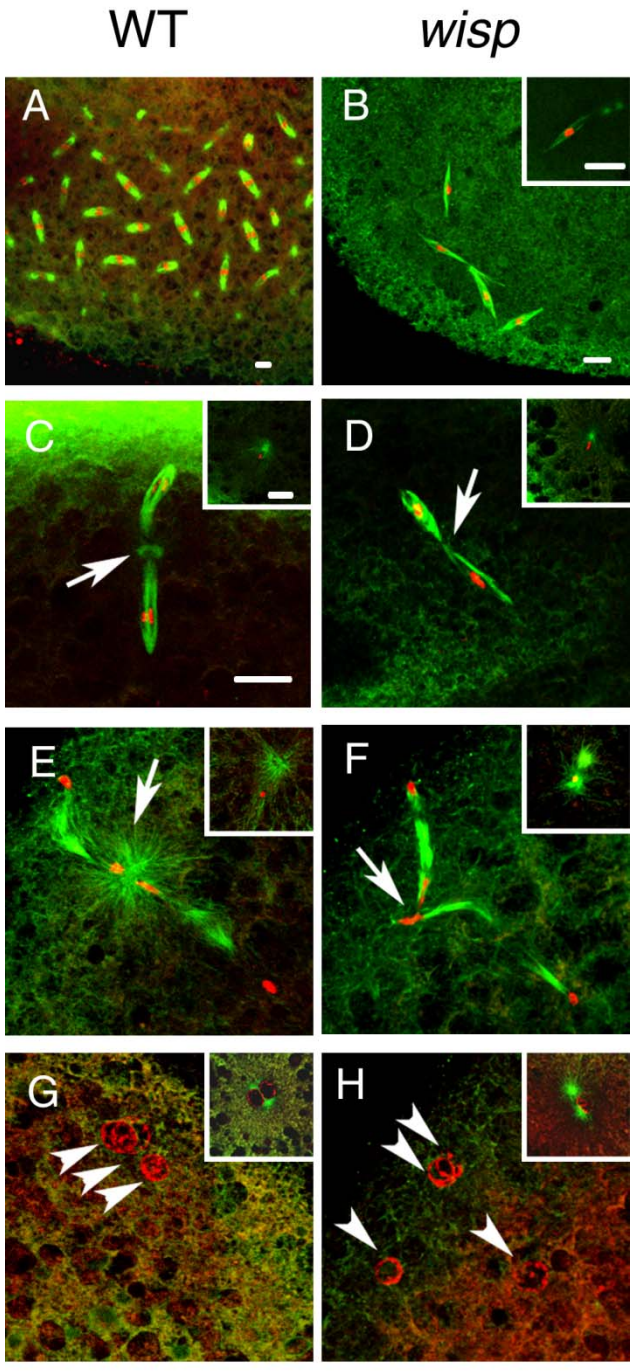


Figure 3.4 Eggs laid by mated *wisp* mutant females are fertilized. Eggs collected at 0- to 2-hr after deposition by $wisp^{41}/wisp^{41}$ mothers were fixed and stained with an anti-sperm tail antibody (1:1000 dilution), a kind gift from T. Karr (University of Bath, UK) (Karr 1991). A sperm tail can be detected in these *wisp*-deficient eggs (arrow). Bar, 10 μm.

Figure 3.5 Fertilized eggs from *wisp* mutant females arrest subsequent to the completion of female meiosis. Embryos derived from *wisp*^{41}*/Df(1)RA47* or *wisp*^{41}/+ control females were fixed and stained with propidium iodide to visualize DNA (red) and with anti-α-tubulin antibody to visualize microtubule (green). Insets show the male nucleus in each embryo. A control 0- to 1-hr old embryo undergoes mitotic divisions (A) while a 0- to 1-hr old embryo from a *wisp*^{41} mutant female is arrested with multiple spindle structures (B). 5- to 15-min embryos were used to observe female meiosis (C-H). Compared to control embryos' metaphase II (C) and telophase II (E), metaphase II (D) and telophase II (F) in *wisp*^{41} mutant embryos have reduced green staining of the acentriolar MTOC between the two linked meiotic spindles (arrows). Female meiosis in control embryos results in four products, three near the surface of the embryo (G, arrowheads) and one apposes to the male nucleus (G inset). All of the four female meiotic products are found near the surface of the *wisp*^{41} mutant embryo (H, arrow heads); none apposed to the male pronucleus (H inset). Bar, 10 μm. D-H are at the same magnification as C.


WT
wisp
A
B
C
D
E
F
G
H

Centrosomes in Drosophila embryos are paternally-derived (Foe et al., 1993), suggesting that the centrosome-containing spindle in *wisp* embryos is associated with the male pronucleus while the other spindles are associated with the four female meiotic products. The arrest phenotype I see for $wisp^{41}$ is like that described for $wisp^{12\text{-}3147}$ (Brent et al., 2000), suggesting that the point mutations I mapped in the latter allele completely disrupt *wisp* function.

Given that *wisp* embryos arrest before embryonic mitosis, I examined whether or not WISP function is essential for fertilized eggs (embryos) to complete female meiosis. I stained 5- to 15-min embryos from either $wisp^{41}$/+ heterozygous control females or $wisp^{41}$/*Df(1)RA47* mutant females and checked the progression of female meiosis after these females were mated to wild type males. All stages of female meiosis are observed in *wisp* mutants (Table 3.2), although one aspect of meiosis appears abnormal. In controls, the two meiosis II spindles are tandemly linked, and bright α-tubulin staining can be seen in an acentriolar MTOC between the two spindles in metaphase II (Figure 3.5C) and telophase II (Figure 3.5E). However in *wisp* mutants, the staining of the acentriolar MTOC between the two meiosis II spindles is greatly diminished (Figures 3.5D and 3.5F).

Although meiosis can complete in *wisp* mutants, I see abnormalities in pronuclear behavior thereafter. In embryos from *wisp* mutant females, the paternal chromosomes decondense to form a male pronucleus (insets of Figures 3.5D, 3.5F and 3.5H), as in control embryos (insets of Figures 3.5C, 3.5E and 3.5G). The aster duplicates in the embryos from *wisp* mutant females (inset Figure 3.5F). However, none of the female meiotic products migrates towards the male pronucleus, and there is thus no pronuclear apposition (Figure 3.5H). Several considerations suggest that the effects of *wisp* mutants on pronuclear migration might reflect *wisp*-mediated control of the synthesis or levels of a microtubule associated protein or a regulator of this

Table 3.2 Meiotic progression in 5-15 min old embryos. Embryos were collected 10 min after deposition by females of the indicated genotype, fixed for 5 min and stained with propidium iodide and anti-α-tubulin antibody to score meiotic and mitotic figures. Stages were determined based on spindle and chromosome configurations.

	Metaphase I	Anaphase I	Metaphase II	Anaphase II
Genotype	No. (%)	No. (%)	No. (%)	No. (%)
wisp^{41}/+	0 (0)	5 (3.9)	16 (12.5)	9 (7.0)
wisp^{41}*/Df(1)RA47*	1 (1.0)	5 (4.9)	11 (12.1)	4 (3.9)
	Telophase II	Pronulei	Mitosis	Arrested
Genotype	No. (%)	No. (%)	No. (%)	No. (%)
wisp^{41}/+	26 (20.3)	23 (10.2)	49 (38.3)	0 (0)
wisp^{41}*/Df(1)RA47*	16 (15.5)	16 (15.5)	0 (0)	50 (48.5)

association. In embryos from *wisp* mutant females, the two centrosome-associated proteins γ-tubulin and CP190 are reduced in the centrosomes compared to wild type, while CP60 is not detected (Brent et al., 2000). Failure of pronuclear migration as observed in *wisp*-deficient embryos also occurs in embryos from *asp* or *KLP3A* mutant females, which lack certain microtubule associated proteins (Riparbelli et al., 2002; Williams et al., 1997). Moreover, defective acentriolar MTOC of meiosis II, reduced sperm aster growth and failure of pronuclear migration, all seen in *wisp*-deficient embryos, have been also reported in mutant embryos lacking POLO, which regulates the organization of microtubule associated structures (Riparbelli et al., 2000).

Wild-type Drosophila eggs can activate without fertilization. Such unfertilized eggs can complete female meiosis but do not develop further (Doane, 1960; Foe et al., 1993). The four haploid nuclei derived from female meiosis retreat to the egg surface and fuse to form a polar body rosette structure; the chromosomes in the rosette are condensed in a mitotic-like arrest (Page and Orr-Weaver, 1997). I examined the four maternally derived nuclei in unfertilized eggs from $wisp^{41}/Df(1)RA47$ females. In control eggs, a polar body rosette structure forms normally (Figures 3.6A and 3.6C). However, the four maternally-derived nuclei in unfertilized *wisp*-deficient eggs become associated with spindles that resemble meiotic spindles (Figure 3.6B). In some cases, the four haploid nuclei fuse to associate with an irregular-sized spindle, but still no rosette-like structure can be observed (Figure 3.6D). These findings suggest that the arrest of these *wisp* mutant eggs is different from the arrest of the polar body nuclei in wild-type unfertilized eggs.

Levels of some active (phospho-) MAPKs are decreased in *wisp* mutant oocytes: Because WISP protein are present both in ovaries and in embryos after egg activation, and because *wisp* mutant oocytes have a defect during the meiosis I arrest prior to egg activation (Brent et al., 2000), my co-authors and I considered the

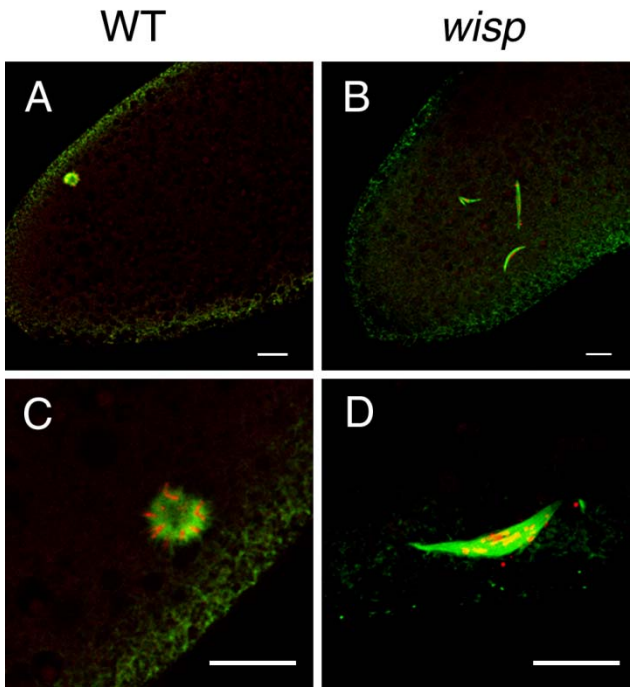


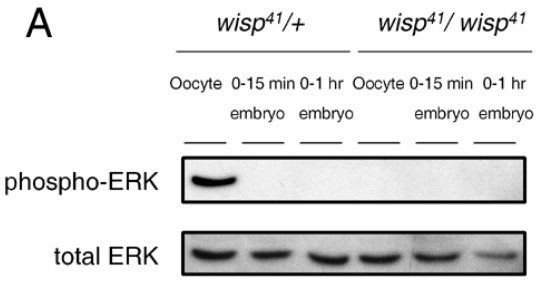
Figure 3.6 Female meiotic products in unfertilized *wisp* eggs. Laid unfertilized eggs from $wisp^{41}$/+ control (A, C) or $wisp^{41}$/*Df(1)RA47* (B, D) mothers were fixed and stained with propidium iodide to visualize DNA (red) and with anti-α-tubulin antibody to visualize microtubule (green). 93% (n=56) of control eggs have formed the polar body rosette. In 100% (n=57) of the eggs from mutant mothers, the female meiotic products associate with anastral spindles (B). In ~45% of these eggs, apposition of multiple female meiotic products can be observed, but no polar rosette is formed (D). Bar, 10 μm.

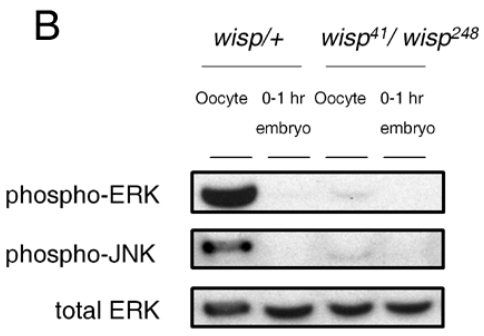
possibility that WISP might have functions in mature oocytes immediately before egg activation. In Drosophila oocytes, levels of active (that is, phosphorylated) MAPKs are high, but upon egg activation (independent of fertilization), the amounts of the active forms of the MAPKs decrease although the total amounts of the MAPK proteins do not change (Sackton et al., 2007). Three classes of MAPK [extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), and p38] are present in Drosophila, as in other organisms. The change in MAPK activity between mature oocytes and activated eggs suggests that MAPK signaling cascades have a necessary function in oocytes and lack of MAPK signaling could be important upon egg activation.

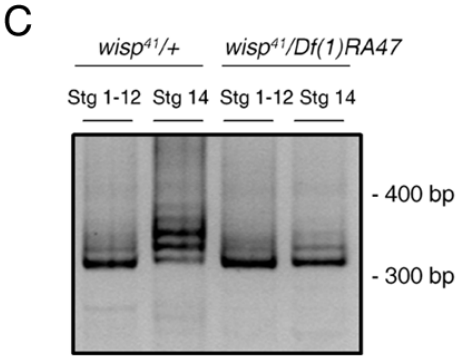
To determine if *wisp* affects MAPK activity at or before egg activation, my co-author K. Sackton examined the amount of phospho-MAPKs in *wisp* mutant oocytes and embryos. In $wisp^{41}$/+ controls (Figure 3.7A, left), the level of phospho-ERK is high in mature (stage 14) oocytes, and decreases upon egg activation as seen previously in wild type (Sackton et al., 2007). In contrast, the level of phospho-ERK in *wisp* mutant mature oocytes is much lower than in control mature oocytes (Figure 3.7A, right). Similarly, stage 14 *wisp* oocytes also contain an unusually low level of phospho-JNK relative to controls (Figure 3.7B), although their levels of phospho-p38 are normal (data not shown). Upon egg activation, the levels of phospho-ERK and phospho-JNK in *wisp* embryos are very low or undetectable, as in wild type embryos (Figures 3.7A and 3.7B). These results suggest that *wisp* plays a role in regulating MAP kinase activity during oogenesis.

Because total ERK protein levels are normal in *wisp* mature oocytes, WISP regulates the activation of this kinase, not its translation. WISP could regulate translation of an upstream activating kinase or kinases in oocytes, so that these kinase(s) in turn can regulate the phosphorylation and activity of ERK and JNK.

Figure 3.7 Levels of phospho-ERK and phospho-JNK, and lengths of *dmos* poly(A) tails, are decreased in *wisp* mutant oocytes. (A) Western blot analysis was performed on total protein extracts of mature (stage 14) ovarian oocytes, 0- to 15-min or 0- to 1-hr embryos from $wisp^{41}/wisp^{41}$ or $wisp^{41}/+$ females. Anti-ERK was used to detect total ERK protein while anti-phospho-ERK was used to detect the active form of ERK. (B) Anti-phospho-JNK was used to detect the active form of JNK in total protein extracts of mature oocytes or 0- to 1-hr embryos from $wisp^{41}/wisp^{248}$ or heterozygous control females. Anti-ERK was used here as a loading control. (C) Stage 1-12 and stage 14 oocytes were hand-dissected from $wisp^{41}/+$ control and $wisp^{41}/Df(1)RA47$ mutant females. PAT assays were performed to examine the poly(A) tail length of *dmos* mRNA. PAT assay products were separated by DNA PAGE. Molecular size markers are shown on the right. In the controls (two lanes on left), the length of *dmos* mRNA poly(A) tail is ~40 nt longer in stage 14 oocytes than in stage 1-12 (two lanes on left), indicating extension of *dmos* mRNA poly(A) tails during oocyte maturation. In contrast, this increase is not seen in *wisp* mutant oocytes (two lanes on right).


A
wisp41/+
wisp41/ wisp41
Oocyte
0-15 min embryo
0-1 hr embryo
Oocyte
0-15 min embryo
0-1 hr embryo
phospho-ERK
total ERK


B
wisp/+
wisp41/ wisp248
Oocyte
0-1 hr embryo
Oocyte
0-1 hr embryo
phospho-ERK
phospho-JNK
total ERK


C
wisp41/+
wisp41/Df(1)RA47
Stg 1-12
Stg 14
Stg 1-12
Stg 14
- 400 bp
- 300 bp

Studies in Xenopus have shown that CPEB-mediated polyadenylation regulates the translation of *mos* mRNA during oocyte maturation (Mendez et al., 2000a), and that MOS phosphorylates MAPK/ERK kinase (MEK),, a kinase that then phosphorylates MAPKs in mature oocytes (Maller et al., 2002). Decreased levels of phospho-ERK and phospho-MEK were observed in oocytes from *dmos* germline clones in Drosophila (Ivanovska et al., 2004). MOS has also been shown to activate JNK in Xenopus eggs and embryos (Bagowski et al., 2001; Mood et al., 2004).

The decreased levels of phospho-ERK and phospho-JNK in *wisp*-deficient Drosophila mature oocytes could be attributable to upstream regulation by DMOS. I explored the possibility that *dmos* transcripts' poly(A) tails could be extended during oocyte maturation, as is the case for Xenopus *mos*, and for Drosophila *cortex* and *cyclin B* transcripts (Benoit et al., 2005; Pesin and Orr-Weaver, 2007), and that *wisp* could mediate this, by analogy to the requirement for xGLD-2 for CPEB-mediated polyadenylation during Xenopus oogenesis (Barnard et al., 2004; Wang et al., 2002). I used PAT assays to examine *dmos* mRNA's poly(A) tail length in immature (stage 1-12) and mature (stage14) oocytes from control females or *wisp* mutant females. In the controls (Figure 3.7C, two lanes on left), *dmos* transcripts have shorter poly(A) tails in stage 1-12 (immature) oocytes; their poly(A) tails are longer by ~40 nt in stage 14 (mature) oocytes. Thus, *dmos* mRNA poly(A) tails lengthen during oocyte maturation in Drosophila (as in Xenopus). In contrast to the situation in controls, the length of the poly(A) tails of *dmos* mRNA in mature oocytes of $wisp^{41}$ mutant females is not significantly different from that in immature oocytes of these females (Figure 3.7C, two lanes on right). Therefore, *wisp* function is needed for poly(A) tail extension on *dmos* mRNA during oocyte maturation.

The shorter poly(A) tail in mature *wisp* oocytes could lead to a failure or inefficiency of *dmos* translation that would in turn explain the decreased levels of

phospho-ERK and phospho-JNK. I unfortunately am unable to examine DMOS protein levels in Drosophila because there is no suitably specific antibody available. However, preliminary data by my co-author indicate that phospho-MEK levels are low in *wisp* mutant mature oocytes (Sackton, unpublished), supporting the hypothesis that MAPK phosphorylation is regulated through the upstream kinases MEK and DMOS during Drosophila oocyte maturation, and that *wisp* controls this by controlling the polyadenylation of *dmos* mRNA (at least) during egg maturation.

Although levels of phospho-ERK and phospho-JNK are low or undetectable in *wisp* mutant mature oocytes, meiosis can still complete upon activation of those eggs. This result is consistent with a previous report that oocytes from *dmos* mutant germline clones have decreased levels of phospho-ERK yet they can complete meiosis (Ivanovska et al., 2004). Although MAPK activity downstream of DMOS is not necessary for completion of meiosis in Drosophila, the regulation of MAPKs or other genes by WISP may be important for other aspects of late oocyte development or for making the oocyte competent to activate.

Conclusions: I have shown that the *wisp* gene encodes a Drosophila member of the GLD-2 cytoplasmic PAP family and the product of this gene is necessary for in several aspects of egg activation and oogenesis. WISP is required for the polyadenylation of *bicoid, Toll*, and *torso* mRNAs upon egg activation. However, translation of SMG, which has been previously reported to correlate with but not depend on polyadenylation of *smg* mRNA upon activation (Tadros et al., 2007), is WISP independent. *wisp* maternal effect mutations lead to very early developmental arrest prior to embryonic mitosis. Eggs lacking maternal WISP function are capable of completing female meiosis to form decondensed female meiotic products. Although the sperm nucleus appears capable of remodeling and forming a male pronucleus in the absence of WISP function, the sperm asters fail to grow and pronuclear migration

does not occur. All the nuclei condense their chromosomes and become associated with a spindle structure, but then arrest at this point. These data are consistent with *wisp*'s being essential for some aspect of the tubulin cytoskeleton in oocytes and embryos. WISP also has functions during oogenesis because levels of active (phospho-) MAPKs are severely reduced in *wisp* oocytes and *dmos* mRNA poly(A) tails fail to extend in *wisp* oocytes during oocyte maturation.

Given its molecular identity, it is likely that WISP affects these aspects of egg activation and oogenesis by a single mechanism: determination of the poly(A) tail length of certain maternal mRNAs. It is likely that there are more mRNA targets of WISP in addition to the ones identified in this study. Future identification of these mRNA targets whose poly(A) tail increase during oogenesis or upon egg activation is *wisp* dependent will help to understand whether and how products of these genes play roles in oogenesis and egg activation.

CHAPTER 4

THOUSANDS OF MATERNAL TRANSCRIPTS ARE REGULATED BY WISP-DEPENDENT CYTOPLASMIC POLYADENYLATION

4.1 Introduction

The transcriptional machinery is largely silent during late oogenesis and early embryogenesis until the activation of the zygotic genome. A large pool of maternal mRNA molecules is accumulated in the oocytes. It is crucial to control the translation of these pre-existing mRNAs for developmental progression. Regulation of mRNA poly(A) tail length in the cytoplasm is a key mechanism that is used to regulate mRNA translation in the developing eggs. Cytoplasmic polyadenylation is controlled by the interaction of proteins and cis-elements in the 3'UTRs of responding mRNAs. Studies in Xenopus have shown that a protein complex, cleavage and polyadenylation specificity factor (CPSF), is involved in polyadenylation by recognizing the hexanucleotide element (Hex), typically AAUAAA, in the 3'UTR (Bilger et al., 1994; Dickson et al., 1999; Sheets et al., 1994). Another factor, cytoplasmic polyadenylation element binding protein (CPEB), recognizes the cytoplasmic polyadenylation element (CPE) and activates the cytoplasmic polyadenylation of many mRNAs during oocyte maturation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). CPEB can also function as a repressor of polyadenylation by recruiting the deadenylase PARN (Kim and Richter, 2006). Other factors, such as Xenopus Pumilio (XPum), can also be recruited to the 3'UTR to repress CPE-mediated polyadenylation (Nakahata et al., 2003).

Cytoplasmic polyadenylation in germ cells is mediated by a special type of poly(A) polymerase, the GLD-2 family proteins (Barnard et al., 2004; Wang et al.,

2002). Drosophila has two GLD-2 family genes, *gld-2* and *wispy (wisp)*, but only the one encoded by the *wisp* gene is highly expressed in female germline cells (Benoit et al., 2008; Cui et al., 2008). WISP function is required for completion of female meiosis and for initiation of embryo development (Benoit et al., 2008; Cui et al., 2008). Fertilized eggs produced by female flies lacking WISP function arrest very early in embryo development with defects in microtubule-based events of female meiosis (Brent et al., 2000). Embryos of *wisp* mutant females also fail to destabilize maternal transcripts (Tadros et al., 2003).

Maternal mRNAs are polyadenylated during Drosophila oogenesis and upon egg activation. WISP, the GLD-2 cytoplasmic poly(A) polymerase, is required for polyadenylation of maternal mRNAs during both of these stages (Benoit et al., 2008; Cui et al., 2008). In oogenesis, *dmos* mRNA is polyadenylated during oocyte maturation and this poly(A) tail elongation is abolished in stage 14 *wisp*-mutant oocytes (Cui et al., 2008). Polyadenylation of mRNAs of the *cortex (cort)* gene, which is required for proper meiotic progression (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Pesin and Orr-Weaver, 2007), also depends on WISP during late oogenesis (Benoit et al., 2008). Subsequent events of development ("egg activation") are triggered by ovulation of the stage 14 oocyte (Heifetz et al., 2001). Egg activation leads to polyadenylation and translation of several maternal mRNAs in the fly embryos (Salles et al., 1994). The lengthening of the poly(A) tails of *bicoid (bcd)*, *torso (tor)* and *Toll (Tl)* mRNAs depends on the function of WISP (Benoit et al., 2008; Cui et al., 2008).

In Chapter 3, I showed that mutant eggs produced from *wisp* mutant females show a wide spectrum of phenotypes. Using the candidate gene approach, I identified several maternal mRNAs whose poly(A) tail lengths greatly decrease in the absence of WISP function. It seems likely that there would be more mRNA targets of WISP

beyond those candidate genes that had been tested, based on prior knowledge that the poly(A) tail length is modulated during oogenesis and upon egg activation. To determine the overall spectrum of WISP-regulated RNAs at these developmental stages, I carried out a microarray analysis. My comparisons of the polyadenylated RNAs in stage 14 oocytes or in activated eggs showed that the polyadenylated portion of the transcriptome is dramatically altered in the absence of WISP function. Thousands of maternal mRNAs depend upon WISP for polyadenylation, with some specifically regulated in oogenesis, some in early embryos, and yet others during both stages. I thus conclude that WISP is a major regulator of polyadenylation of maternal mRNAs.

4.2 Materials and Methods

Drosophila stocks and sample collection: Male flies carrying the *wisp* null allele, $wisp^{41}$ (Cui et al., 2008) were crossed to *Df(1)RA47/*FM7c female flies. $wisp^{41}$*/FM7c* (control) or $wisp^{41}$*/Df* (*wisp*-deficient) virgin female progeny were separated from males and aged on standard yeast/glucose medium until use. Stage 14 oocytes were hand dissected from 3 to 5-day-old virgin females in hypertonic isolation buffer (Page and Orr-Weaver, 1997), which does not activate eggs. Virgin females that were 3- to 4-days-old were mated to wild-type Oregon-R males and embryos were collected 0- to 1-hr post egg deposition. Hereafter, "0- to 1-hr embryos" will refer to fertilized eggs collected 0- to 1-hr post egg deposition.

Sample preparation and hybridizing of microarrays: For each sample, total RNA was extracted from ~2000 stage 14 oocytes or 0- to 1-hr embryos using the TRIzol reagent (Invitrogen, Carlsbad, CA). Polyadenylated RNA was then isolated from total RNA using the Oligotex mRNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized from 40 μg of total RNA or 800 ng poly(A)-selected mRNA in

a 200 µl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.375 mM dATP, 0.375 mM dCTP, 0.375 mM dGTP, 0.225 mM dTTP, 0.05 mM aminoallyl-dUTP. 50 µg dN_9 primer, and 20 ng murine Moloney leukemia virus (M-MLV) reverse transcriptase. Reactions were incubated at 42 °C for at least 2 hr and then cDNAs were purified using a Zymo25 DNA purification column (Zymo Research, Orange, CA).

The purified cDNA was divided into halves. One half was conjugated to Cy3 and the other to Cy5 fluorescent dye (GE Healthcare, Piscataway, NJ) to generate dye-swap pairs. Dye conjugation was performed at 60 °C for 60 min in a 10 µl reaction containing 50 mM sodium bicarbonate (pH 9.0), 50% DMSO, and ~20 µg of NHS-derivatized fluorophore. Labeled cDNA was purified using a Zymo25 DNA purification column.

Drosophila Oligo microarrays (Agilent Design ID 18972, Agilent, Foster City, CA) were hybridized with labeled cDNA in 1X Hi-RPM Hybridization buffer (Agilent) at 60 °C for 16 hr. Four groups of comparisons were performed: WT vs. *wisp* total RNA from oocytes, WT vs. *wisp* total RNA from fertilized eggs, WT vs. *wisp* poly(A)-selected RNA from oocytes, WT vs. *wisp* poly(A)-selected RNA from fertilized eggs. Each comparison consisted of three independent RNA extractions and each experiment was done with in dye-swap pairs as two technical replicates.

Image processing and data analysis: Microarray images were acquired using an Axon GenePix 4000B scanner reading at wavelengths of 635 nm and 532 nm and then pre-processed in GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). Raw fluorescence intensities for the 635 nm and 532 nm images of each spot were transformed into log2 ratio values. Data from spots whose fluorescence intensity was lower than the mean background level of the array were removed from further analysis.

Data for each probe were first averaged between dye-swap pairs and then averaged among duplicated probes in the same experiment.

For stage 14 oocytes, a total of 7889 probe sets, representing 6124 transcripts were detected in all three total RNA comparisons and all three polyadenylated RNA comparisons. For 0- to 1-hr embryos, 6943 probe sets, representing 5446 transcripts were detected in all three total RNA comparisons and all three polyadenylated RNA comparisons. The intensity ratio of each probe was normalized using the mean ratio of probes for the ribosomal protein components detected on the array, 22 probes for stage 14 oocytes and 21 probes for 0- to 1-hr embryo, respectively. Statistical significance was assessed as the test-wise *P* values.

Lists of genes were tested by the DAVID Functional Annotation Bioinformatics Microarray Analysis (http://david.abcc.ncifcrf.gov/) to reveal over-represented Gene Ontology (GO) groups of annotated genes (Dennis et al., 2003).

Quantitative PCR and poly(A) tail assay: Total RNA was prepared as described above. Synthesis of cDNA was done using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed using an ABI Prism 7000 system (Applied Biosystems, Foster City, CA). Each reaction was prepared in a 25 µl mixture containing 5 µl cDNA template, 0.2 µM of each primer and 1X SYBR Green Super Mix (Applied Biosystems).

PCR-based polyA tail (PAT) assay was performed as previously described (Cui et al., 2008). PCR was performed on the PAT cDNAs using a gene-specific primer and the oligo$(dT)_{12}$-anchor to assess the length of the poly(A) tail of a specific mRNA. PCR products from PAT were separated on 8% acrylamide gels.

Cross-linking and RNA immunoprecipitation: Early embryos (0- to 1-hr post deposition) were collected from Oregon-R P2 flies as previously described. These

embryos were permeabilized and cross-linked by shaking at room temperature in a 1:3 mixture of 1.8% formaldehyde cross-linking solution [50 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1.8% Formaldehyde] and heptane. Cross-linked embryos were then washed in 1 X PBST with 125 mM glycine and homogenized in the homogenization buffer [10 mM HEPES (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40 (NP-40, Sigma), 1 mM dithiothreitol (DTT), 100 U/ml RNasin RNase inhibitor (Promega), 2 mM vanadyl ribonucleoside complexes (VRC, Sigma), protease inhibitor cocktail (Roche)] at 4°C. Lysates were filtered using Miracloth (EMD Chemicals, Gibbstown, NJ) and then centrifuged at 1,500 g for 10 minutes at 4°C. Protein concentration of supernatants was adjusted to 2 mg/ml by diluting in the immunoprecipitation buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40, 1 mM DTT, 40 U/ml RNasin RNase inhibitor, 2 mM VRC, protease inhibitor cocktail].

Protein A Sepharose beads (Sigma) were washed in the immunoprecipitation buffer with gentle shaking and centrifugation for 30 sec at 1,500 g. Egg extracts were pre-cleared with 50 µl of beads for 1 hr at 4°C, centrifuged at 1,500 g for 30 sec. The supernatant containing 5 mg of total proteins was incubated with 10 µg of anti-WISP antibody or pre-immune serum (as control) and 50 µl of clean beads at 4°C with gentle rotating for 16 hr. The beads were collected with centrifugation and washed four times with the immunoprecipitation buffer and then four times with the immunoprecipitation buffer supplied with 1 M Urea.

Beads were incubated in the elution buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 40 U/ml RNasin RNase inhibitor] for 10 min with vortexing at 37°C and centrifuged at 1,500 g for 30 sec. The supernatants were treated with proteinase K (Roche) for 1 hr at 42°C and incubated for 1 hr at 65°C to reverse the cross-links. RNA was purified using Trizol as described above.

4.3 Results and Discussion

Overview of the microarray experiments

Using a candidate gene approach in Chapter 3, I identified specific maternal mRNAs whose poly(A) tail lengths were regulated by WISP in oocytes and embryos (Cui et al., 2008). To identify globally the maternal mRNAs whose poly(A) tail lengths are regulated by WISP, I used microarray analysis to directly compare directly the poly(A) selected transcriptome of *wisp* deficient oocytes and embryos with that of normal control oocytes and embryos.

Total RNA was extracted from dissected stage 14 (mature) oocytes or fertilized eggs collected at 0- to 1-hr post egg deposition (hereafter as "0- to 1-hr embryos") produced by wild type control females or *wisp* mutant females. Each RNA sample was divided into halves. One half was subjected to the commercially available poly(A)-selection method to enrich for polyadenylated RNAs. The poly(A)-selected RNAs were then used to prepare cDNA probes labeled with either Cy3 or Cy5 fluorophores ("poly(A) RNA"). The other half of each RNA sample was used directly to prepare Cy3 or Cy5 labeled cDNA probes without any poly(A) selection ("total RNA"). cDNAs of wild type (WT) and corresponding *wisp*-deficient (*wisp*) samples labeled with different fluorophores were then hybridized in the same reaction to an Agilent Drosophila Oligonucleotide microarray (Agilent Design ID 18972).

Four groups of comparisons were performed using microarrays: WT vs. *wisp* total RNA from oocytes, WT vs. *wisp* poly(A)-selected RNA from oocytes, WT vs. *wisp* total RNA from early embryos, WT vs. *wisp* poly(A)-selected RNA from early embryos. For the sets using non-poly(A) selected RNAs, the ratio of signals reflects the difference in abundance of the total RNA for a given transcript between wild type and *wisp* mutant, regardless of its poly(A) status. For the sets using the poy(A)-selected RNAs, the ratio of signals reflects the difference in abundance of the poly(A)-

selected RNA for a given transcript between wild type and *wisp* mutant. For each developmental stage, only probes with data from all three replicates of poly(A)-selected RNA comparisons and all three replicates of non-poly(A) selected RNA comparisons were used in subsequent analysis.

To normalize the microarray data, I used the signals for the probe sets specific for ribosome constituent mRNAs as these mRNAs are expected to be stable during oogenesis and early embryogenesis (Tadros et al., 2007) and the length of their poly(A) tails is not affected in the *wisp* mutant (Benoit et al., 2008; Cui et al., 2008).

Polyadenylated transcriptome is altered in the absence of WISP function

Stage 14 oocytes: To identify target mRNAs whose poly(A) tail elongation is *wisp*-dependent in stage 14 oocytes, I used microarray analysis to compare the total vs. poly(A)-selected RNA populations of stage 14 oocytes produced by wild type females and *wisp* females. As shown in Figure 4.1A, 7889 probe sets were detected in all six experiments using stage 14 oocytes, including three total comparisons and three poly(A)-selected RNA comparisons. After analyzing the data, I found that the total transcriptome showed a slight increase in abundance in *wisp* oocytes compared to wild type oocytes, with a median change of 1.2-fold among all the probe sets. This indicates that some transcripts may be accumulated in the *wisp* oocytes.

In contrast, I observed a significant decrease in abundance of the poly(A)-selected RNA population in *wisp* oocytes compare to normal stage 14 oocytes. Among the 7889 probe sets, the median change in abundance of poly(A)-selected RNAs showed a 2.2-fold decrease in *wisp* oocytes compare to control oocytes.

Since the average abundance of total transcripts in WT and *wisp* oocytes are similar, the decrease in abundance of many poly(A)-selected RNAs in *wisp* oocytes could not be due to a general destabilization of the transcripts in the *wisp* mutant. Instead, it is mostly likely due to a dramatic decrease of the proportion of RNAs with

Figure 4.1 Summary of microarray results. Columns represent individual experiments, three biological replicates of total RNA comparison and three biological replicates of poly(A) selected RNA comparison of stage 14 oocytes (A) and 0- to 1-hr embryos (B). Rows represent probe sets. Green-to-red color code indicates the scale of fold change in RNA abundance in wild type oocytes compared to *wisp* deficient oocytes. Probe sets are ordered from top to bottom based on the fold change of poly(A) selected RNA abundance between wild type and *wisp* deficient samples.


A
Oocyte (7889 probes)
Total
PolyA+
ratio (wild type vs. wisp)
< 1/16
1:1
> 16
B
Embryo (6943 probes)
Total
PolyA+
ratio (wild type vs. wisp)
< 1/16
1:1
> 16

long poly(A) tails. Thus many maternal transcripts are likely not polyadenylated or less polyadenylated in the absence of WISP function.

The slight accumulation of total transcripts in *wisp* oocytes maybe a secondary effect of the *wisp* mutation and for the purpose of this study I did not investigate this further. I will discuss RNA degradation in Chapter 6.

Early embryos: I performed the same type of analysis as described above on total and poly(A)-selected RNA populations from 0- to 1-hr embryos. As shown in Figure 4.1B, 6943 probe sets were detected in all six experiments using 0- to 1-hr embryos, including three WT vs. *wisp* total RNA comparisons and three WT vs. *wisp* poly(A)-selected RNA comparisons. Analysis of the embryo data showed similar but not identical results to those for the stage 14 oocytes. Like that for the stage 14 oocytes, among all the detected probe sets *wisp* embryos showed a median 4.4-fold decrease in the abundance of poly(A)-selected RNAs as compared to wild type. Unlike that for the stage 14 *wisp* oocytes, *wisp* embryos showed a median 1.1-fold decrease in the abundance of the total RNA population as compared to wild type. Even after normalizing the data against the change of total RNA abundance, *wisp* mutant embryos still showed a more dramatic decrease in the abundance of the poly(A) selected RNA population as compared to WT controls.

The fold change in abundance of the poly(A)-selected RNAs between *wisp* deficient and wild type embryos was greater than that between *wisp* deficient and wild type oocytes. This difference could be due to additional polyadenylation of mRNAs that happening at egg activation, as WISP also plays a role in cytoplasmic polyadenylation that is triggered upon egg activation (Benoit et al., 2008; Cui et al., 2008). I have previously found that the poly(A) tail of *bcd* mRNA was further shortened in *wisp* mutant embryos compared to *wisp* mutant oocytes (Cui et al., 2008). Thus it is very likely that some other mRNAs exhibit further shortening of their

poly(A) tails in *wisp* deficient embryos. I think that the defects of the polyA+ status observed in the early embryos could reflect an accumulative effect of polyadenylation defects at both oocyte maturation and egg activation.

Different from the slight accumulation of total transcripts that I observed in *wisp* oocytes, *wisp* embryos showed a median 1.1 fold decrease in total RNA abundance among all the probe sets. This suggests that egg activation probably activates a RNA degradation pathway and this pathway does not depend on cytoplasmic polyadenylation. The RNAi pathway is not active in Drosophila mature oocytes but is activated during egg activation, leading to RNA degradation (Kennerdell et al., 2002). One possibility is that this RNAi pathway causes degradation of some maternal transcripts in *wisp* deficient embryos.

Identification of specific mRNAs whose poly(A) tail length depends on WISP function

Stage 14 oocytes: I next wanted to identify those maternal transcripts whose poly(A) tails are shortened in the *wisp* mutant. For a given mRNA, I calculated the mean ratio of WT vs. *wisp* for its poly(A)-selected RNA abundance (A-ratio) and the mean ratio of WT vs. *wisp* for its total RNA abundance (T-ratio). I then compared these two ratios and determined the poly(A) index (A-ratio divided by T-ratio) for each mRNA transcript. If the poly(A) tail of a given mRNA is not affected in *wisp* mutant, then the two ratios for this mRNA are expected to be similar and its poly(A) index is expected to be 1. However, if a mRNA depends on WISP function for its polyadenylation, the poly(A) index for that mRNA is expected to be greater than 1. For a given mRNA, I also used the two-group t-test to estimate the significance of difference between the ratios of WT vs. *wisp* for its poly(A)-selected RNA abundance and ratios of WT vs. *wisp* for its total RNA abundance.

Based on these analyses, I found that 4053 probes, or about 51% of all the 7889 probe sets detected on the oocyte microarrays, showed a poly(A) index of 2 or more (more than two-fold difference between the A-ratio and T-ratio) ($P < 0.01$ based on a two-group t-test) (Figure 4.2A). I assigned all these mRNAs as putative WISP targets in the stage 14 oocytes.

Previous studies have reported that 36 maternal mRNAs have short poly(A) tails in *wisp* mutant oocytes (Benoit et al., 2008; Cui et al., 2008). Among them, I identified 23 of these 36 in my analysis (listed in Table 4.1). For the other 13: (1) Probes for 5 genes (*oskar*, *cortex*, *cdc6*, *cup*, and *Decondensation factor 31*) showed a poly(A) index of > 2 but with a *P* value ranging from 0.011 to 0.041. They were determined as not being WISP regulated due to the stringent *P* value cut-off that I used (< 0.01). (2) Probes for 2 genes (*CG15092* and *modulo*) both showed a >2-fold decrease in total transcript abundance in *wisp* oocytes. This suggests that poly(A) tail shortening of these two transcripts probably triggers degradation of their RNAs. I did not identify them because I looked for transcripts whose total and poly(A)-selected forms were affected differently by *wisp* mutant. (3) Probe for 1 gene (*bicaudal*) did not show any significant change in both types of RNA abundance between *wisp* and wild type oocytes. (4) Probes for 5 genes (*nanos*, *arrest*, *CG9742*, *deadhead*, and *Histone H4*) were filtered out during data processing and were not in the final data set that I analyzed.

For subsequent analysis, I used five of the known WISP targets as the positive control. Positions of probes specific for these five known targets were shown in the map on the right in Figure 4.2A.

I next chose eight putative WISP targets identified above and used real-time quantitative PCR (qPCR) to validate the microarray results. These eight mRNAs were chosen from the list of putative wisp targets with a range of *P* values, from the 10th

Figure 4.2 WISP targets in stage 14 oocytes. (A) Specific probe sets (4053) representing RNAs whose polyadenylation was altered in *wisp* deficient oocytes. Columns represent individual experiments, three biological replicates of total RNA comparison and three biological replicates of poly(A) selected RNA comparison. Rows represent probe sets. Green-to-red color code indicates the scale of fold change in RNA abundance in wild type oocytes compared to *wisp* deficient oocytes. Probe sets are ordered from top to bottom based on *P* values between the total RNA difference in abundance and poly(A) selected RNA difference in abundance. Arrowheads indicate probes for previously identified RNAs that are dependent on WISP for polyadenylation. Bars indicate probes for putative targets validated in (B) and (C). (B) qPCR validation. The poly(A) selected RNA abundance is shown as a proportion of the total RNA abundance. Twelve mRNAs were tested, including two known targets that have been shown in previous studies to be regulated, eight putative WISP targets, two non-targets whose abundance is not affected in *wisp* deficient oocytes. All data were normalized to *RpL32*. Results from wild type oocytes (blue bars) and from *wisp* deficient oocytes (red bars) are shown for two biological replicates and one technical replicate. Error bars indicate standard deviation. (C) PAT assays. Poly(A) tail length of 18 mRNAs was assessed using gene-specific primers in PAT assays, including four ribosomal protein mRNAs, four known targets that have been tested previously in *wisp* mutant oocytes, and ten putative WISP targets predicted by the microarray data. Bars indicate the longest products produced by PAT.

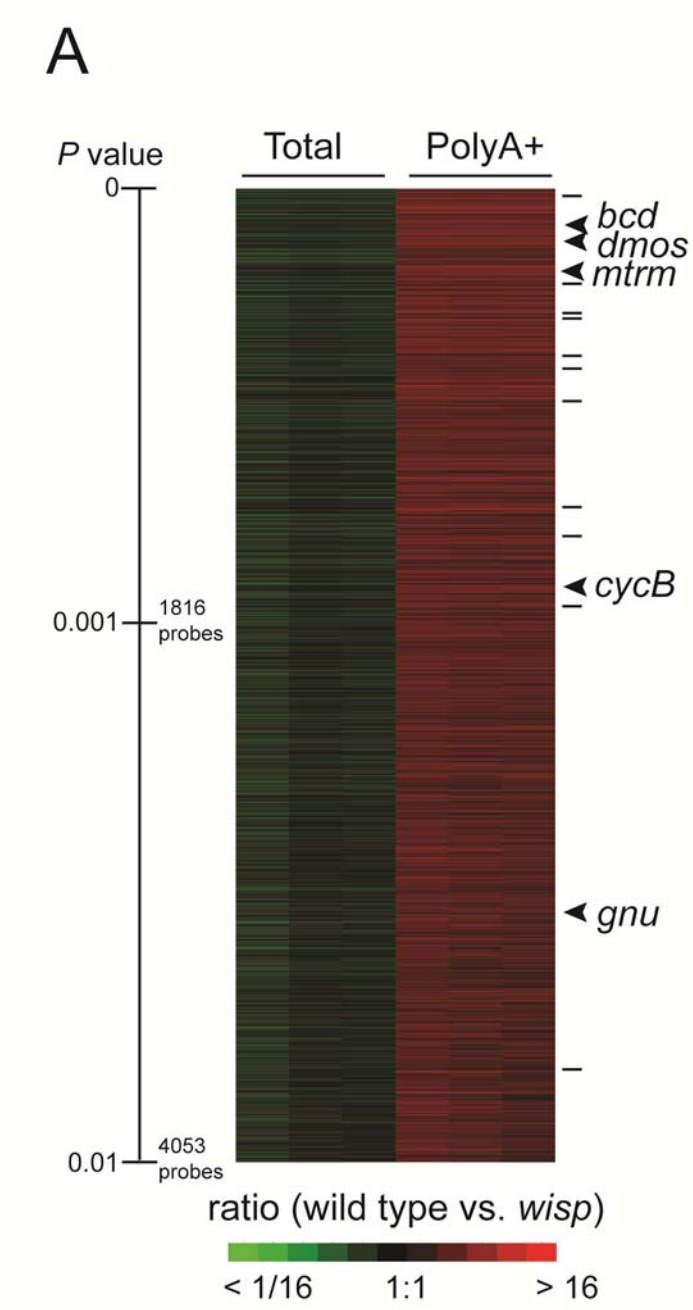

A
P value
0
0.001
1816
probes
0.01
4053
probes
Total
PolyA+
bcd
dmos
mtrm
cycB
gnu
ratio (wild type vs. wisp)
< 1/16
1:1
> 16

Figure 4.2 (Continued)

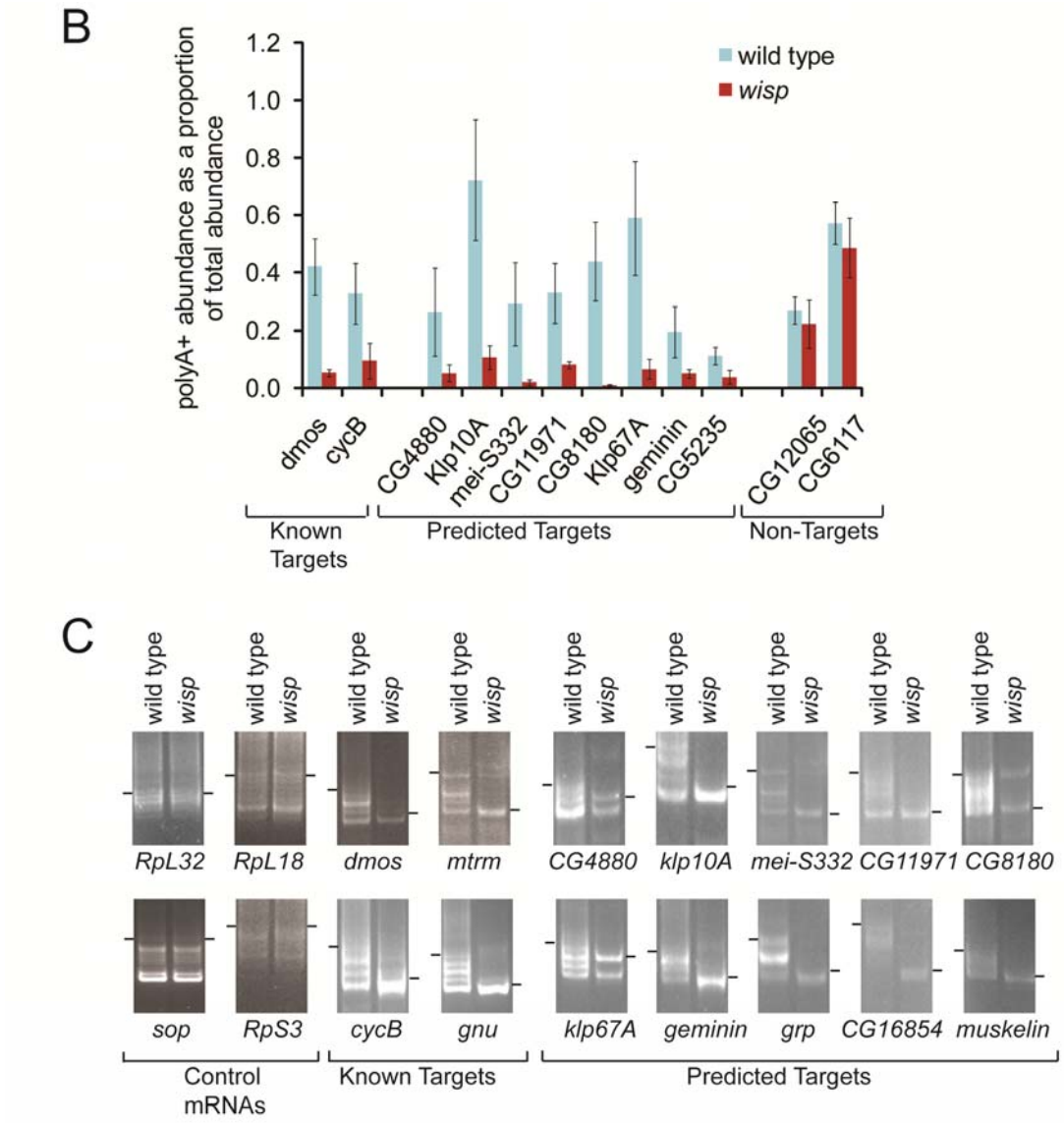


Table 4.1 List of known WISP targets in oocytes that were identified in the microarray analysis.

Gene name	Gene name
bicoid	*CG10377*
dmos	*Minichromosome maintenance 5*
cyclin B	*Protein on ecdysone puffs*
arc-p34	*pleiohomeotic like*
cdc2	*smt3*
caudal	*Set*
CG3800	*giant nuclei*
CG7033	*Bicaudal-C*
CG7101	*cdc2c*
CG17018	*Regulator of cyclin A1*
Histone H2A variant	*matrimony*
CG8975	

(*CG4880, P* = 0.000007) to the 3735th (*CG5235, P* = 0.007) (*CG4880*, *klp10A*, *mei-S332*, *CG11971*, *CG8180*, *klp67A*, *geminin*, and *CG5235*). Two mRNAs whose poly(A) index was <1 were used as negative controls. Two previously known WISP targets, *dmos* and *cyclin B,* were used as positive controls. I used qPCR to assess the abundance of poly(A) RNA vs. total RNA for these mRNAs in WT and *wisp* oocytes, and plotted the ratio of poly(A)-selected RNA/total RNA for each transcript. As shown in Figure 4.2B, all eight putative targets and both positive controls, but not the two negative controls (*CG12065* and *CG6117*), showed a decreased ratio of poly(A)-selected RNA/total RNA in *wisp* mutant compared to wild type, suggesting that *wisp* mutant affected the poly(A) selection of these mRNAs.

I then directly examined the length of poly(A) tails on these putative targets using a PCR-based poly(A)-tail (PAT) test (Salles et al., 1994). A total of ten putative targets with a range of *P* values, from the 10th (*CG4880, P* = 0.000007) to the 1686th *P* value (*geminin, P* = 0.0008) were tested using PAT. I could not detect specific bands for *CG5235* in PAT due to technical difficulties, so I chose three additional putative targets (*grapes*, *CG16854*, and *muskelin*) to test. Four previous known WISP targets (*dmos*, *matrimony*, *cyclin B*, and *giant nuclei*) were used as positive controls. Negative controls were also included as the poly(A) tail length of four mRNAs encoding ribosome proteins (*sop, RpL32, RpL18, RpS3*) was not affected in *wisp*-deficient oocytes. As shown in Figure 4.2C in *wisp* deficient oocytes, the poly(A) tail lengths of both positive controls and the test mRNAs were shortened for ~ 40 to ~120 nt depending on the specific mRNA. I noticed that in *wisp* deficient oocytes all the tested mRNAs still have a short poly(A) tail, ~10 to ~40 nt depending on the specific mRNA. I believe this residual tail is probably due to the function of nuclear poly(A) polymerase, encoded by the *hiiragi* gene, which functions in early oogenesis (Benoit et al., 2008). The PAT results suggest that the array data faithfully represent maternal

transcripts whose poly(A) tail elongation depends on WISP function in stage 14 oocytes.

Early embryos: Using the same type of analysis, 3849 probe sets, about 55% of the 6943 probe sets detected on the microarray, were determined as putative *wisp* targets (Figure 4.3A). Five mRNAs, *bicoid, torso, Toll, oskar, and nanos,* have been shown previously to be affected in *wisp* deficient embryos (Benoit et al., 2008; Cui et al., 2008). Positions of probes specific for these five previously known WISP targets are shown in the map on the right in Figure 4.3A.

I chose nine putative WISP targets identified above and used the same qPCR method as in Figure 4.2B to validate the microarray results from embryos. These nine mRNAs were chosen from the list of putative wisp targets with a range of *P* values from the 198th (*cyclin A, P* = 0.0002) to the 2291th (*CG5784, P* = 0.008) (*cyclin A*, *grapes*, *Bj1*, *CG8180*, *pimples*, *string*, *smaug*, *ubcE2h*, and *CG5784*). *CG3083* was chosen as negative control. Two previously known targets, *bicoid* and *Toll* were used as positive controls in the experiment. As shown in Figure 4.3B, all nine putative targets and two positive controls, but not the negative control *CG3083*, showed a decreased ratio of poly(A)-selected RNA/total RNA in wisp mutant compared to wild type, suggesting that *wisp* mutant affected the poly(A) selection of these mRNAs.

I also used the PAT assays to examine the length of the poly(A) tails on selected mRNAs (Figure 4.3B). I could not detect specific bands for four of the targets (*string*, *smaug*, *ubcE2h*, *CG5784*) in PAT due to technical difficulties, so I chose five additional putative targets (*CG11844*, *CG14464*, *CG7627*, *CG1836*, and *moira*) to test. A total of ten mRNAs were chosen from the list of putative targets with a range of *P* values, from the 198th (*cyclin A, P* = 0.0002) to the 2208th *P* value (*moira, P* = 0.003). Two previously known *wisp* targets, *bicoid* and *Toll,* were used as positive controls. As shown in Figure 4.3C, the poly(A) tail of both the positive controls and the

Figure 4.3 WISP targets in early (0-1hr old) embryos. (A) Specific probe sets (3849) representing RNAs whose polyadenylation was altered in *wisp* deficient embryos. Columns represent individual experiments, three biological replicates of total RNA comparison and three biological replicates of poly(A) selected RNA comparison. Rows represent probe sets. Green-to-red color code indicates the scale of fold change in RNA abundance in wild type embryos compared to *wisp* deficient embryos. Probe sets are ordered from top to bottom based on *P* values between the total RNA difference in abundance and poly(A) selected RNA difference in abundance. Arrowheads indicate probes for previously identified RNAs that are dependent on WISP for polyadenylation. Bars indicate probes for putative targets validated in (B) and (C). (B) qPCR validation. The poly(A) selected RNA abundance is shown as a proportion of the total RNA abundance. Twelve mRNAs were tested, including two known targets that have been shown in previous studies to be regulated, nine putative targets, one non-target whose abundance is not affected in *wisp* deficient embryos. All data were normalized to *RpL32*. Results from wild type embryos (blue bars) and from *wisp* deficient embryos (red bars) are shown for two biological replicates and one technical replicate. Error bars indicate standard deviation. (C) PAT assays. Poly(A) tail length of 16 mRNAs was assessed using gene-specific primers in PAT assays, including four ribosomal protein mRNAs, two known targets that have been tested previously in *wisp* deficient embryos, and ten putative WISP targets predicted by the microarray data. Bars indicate the longest products produced by PAT. Two sets of bars mark the results for *cycA* and *Bj1* because these mRNAs gave two sets of PAT products, probably results from poly(A) tails of two isoforms of the transcripts.

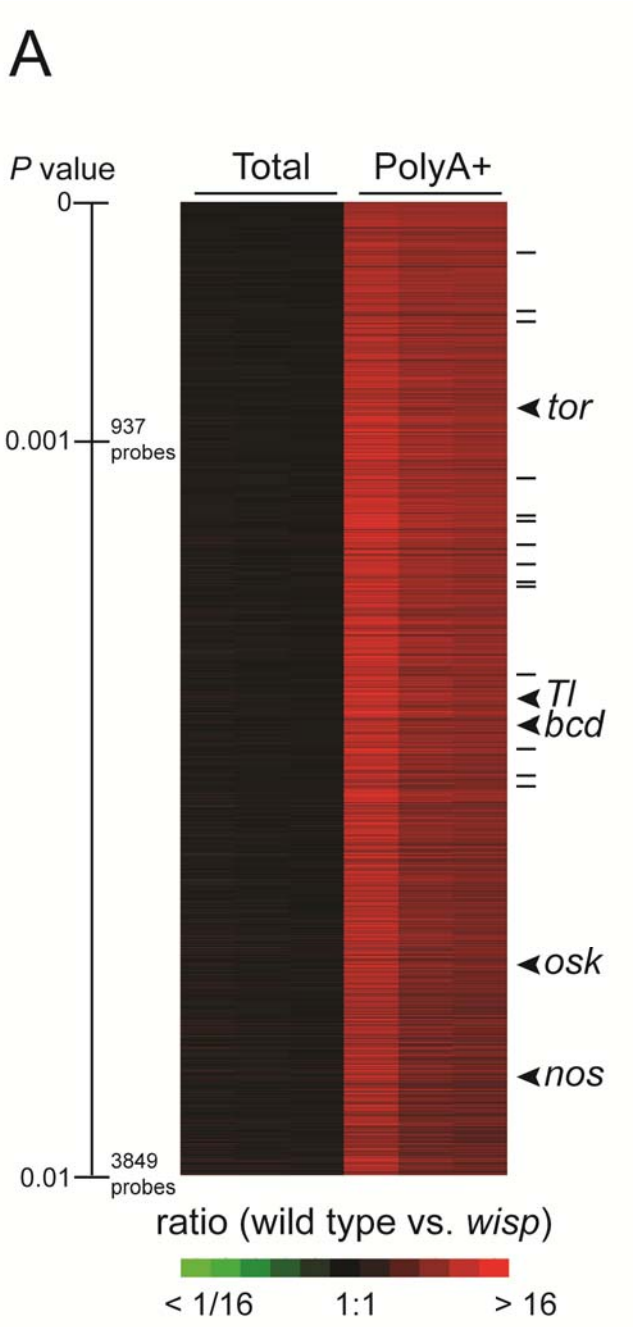
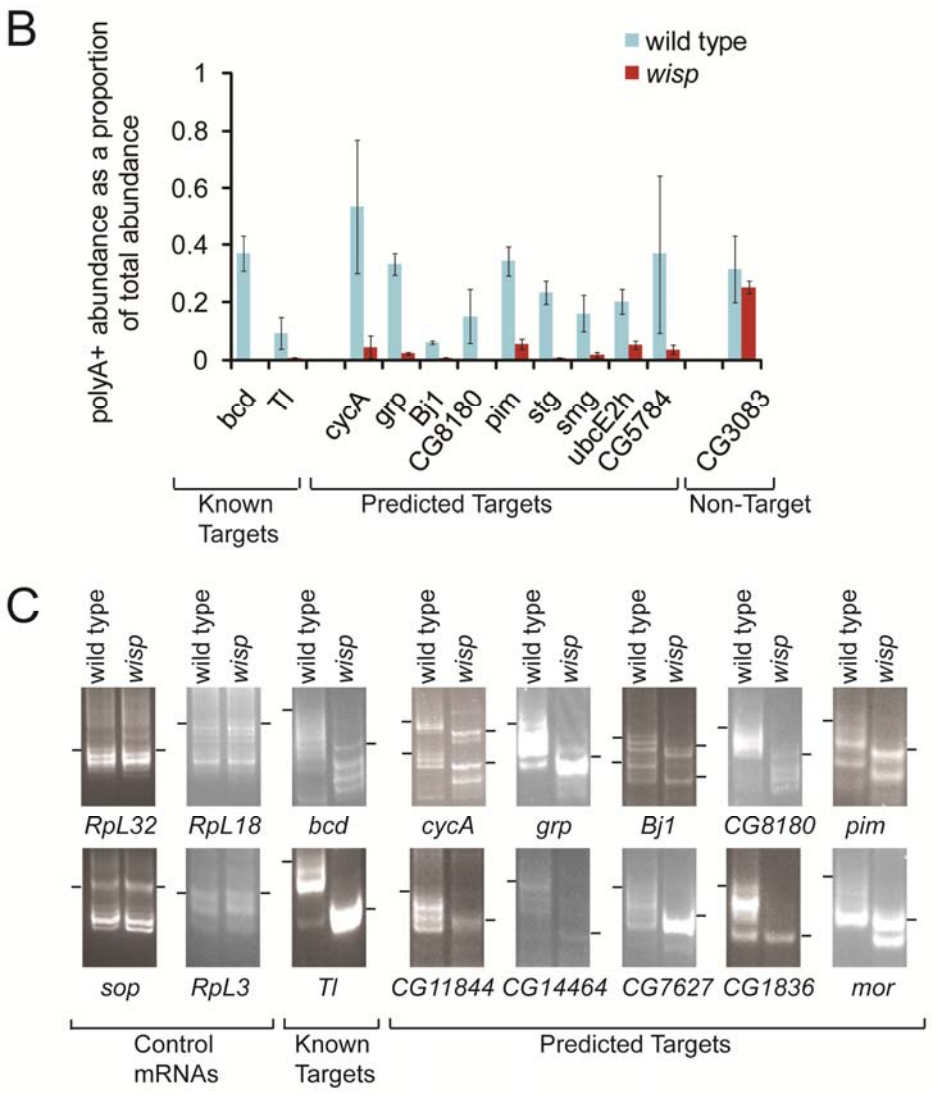

A
P value
0
0.001
937
probes
0.01
3849
probes
Total
PolyA+
tor
Tl
bcd
osk
nos
ratio (wild type vs. wisp)
< 1/16
1:1
> 16

Figure 4.3 (Continued)



predicted WISP targets were shortened in *wisp* deficient embryos. Four negative controls were also included as the poly(A) tail length of four mRNAs encoding ribosome proteins (*sop, RpL32, RpL18, RpL3*) was not affected in *wisp*-deficient oocytes (Figure 4.3C).

In both *wisp* oocytes and embryos, I found that poly(A) tails of many maternal transcripts are affected, suggesting that cytoplasmic polyadenylation is a major mechanism regulating maternal transcripts during oocyte and embryo development.

Functional relevance of genes targeted by WISP in stage 14 oocytes

To address the question of whether some functional groups of genes are over-represented in the identified targets, I did Gene Ontology (GO) annotation analysis using DAVID (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003). This analysis reveals over-represented functional groups of genes in the list of putative WISP targets.

The 4053 probe sets that were identified as WISP targets in stage 14 oocytes collapsed into 3301 unique genes. GO analysis revealed that "cell cycle" related genes were represented by 3 of the top ten categories within Biological Process (*P* values from 2.12E-6 to 4.18E-6) (Table 4.2). These groups include genes that encode cell cycle regulators, such as cyclins (cyclin A, cyclin B, cyclin B3, cyclin D and cyclin E) and proteins that are known to be involved in meiosis. The enrichment of cell cycle related genes can be linked to the phenotypes observed in oocytes produced from *wisp* mutant mothers. One such example is *klp3A,* encoding a kinesin-like protein which is required for pronuclear migration at the end of meiosis (Williams et al., 1997). Previously I observed that the female pronucleus does not migrate in *wisp* mutant (Cui et al., 2008). This phenotype could be due to the lack of translation of *klp3A* mRNA when its poly(A) tail shortened in the absence of WISP function.

Using the KEGG pathway identifier in GO analysis, I found that members of the ubiquitination machinery were highly enriched in the WISP target groups in

Table 4.2 Over-representation of the gene categories shown by DAVID analysis of *wisp* targets in stage 14 oocytes.

Term	Count	*P* value
Biological process		
GO:0010605~negative regulation of macromolecule metabolic process	125	3.89E-07
GO:0048285~organelle fission	82	1.65E-06
GO:0000087~M phase of mitotic cell cycle	79	2.12E-06
GO:0051172~negative regulation of nitrogen compound metabolic process	81	2.31E-06
GO:0000280~nuclear division	78	2.98E-06
GO:0007067~mitosis	77	4.18E-06
GO:0009890~negative regulation of biosynthetic process	89	1.13E-05
GO:0051253~negative regulation of RNA metabolic process	69	1.37E-05
GO:0007423~sensory organ development	136	1.88E-05
GO:0016481~negative regulation of transcription	70	4.16E-05
GO:0001654~eye development	108	4.52E-05
GO:0010629~negative regulation of gene expression	94	5.01E-05
GO:0048749~compound eye development	103	6.11E-05
GO:0045892~negative regulation of transcription, DNA-dependent	63	9.82E-05
GO:0030182~neuron differentiation	162	1.66E-04
GO:0007049~cell cycle	221	2.24E-04
GO:0070727~cellular macromolecule localization	109	2.27E-04
GO:0048610~reproductive cellular process	187	2.56E-04
GO:0019953~sexual reproduction	243	3.38E-04
GO:0007276~gamete generation	239	3.82E-04
GO:0007281~germ cell development	101	4.07E-04
GO:0048592~eye morphogenesis	86	4.15E-04
GO:0007264~small GTPase mediated signal transduction	58	4.63E-04
GO:0007059~chromosome segregation	64	4.81E-04
GO:0009994~oocyte differentiation	64	4.81E-04
Cellular compartment		
GO:0005694~chromosome	129	1.93E-05
GO:0044427~chromosomal part	103	2.04E-04
Molecular Function		
GO:0003702~RNA polymerase II transcription factor activity	94	1.17E-04
GO:0003677~DNA binding	287	3.53E-04

oocytes (P = 8.9E-7). Transcripts of 63 genes in this pathway were detected on the microarray and 53 were identified as regulated by WISP. These 53 genes include 2 E1 ubiquitin-activating enzymes, 14 E2 ubiquitin-conjugating enzymes and most subunits of the two major multi subunit E3 ubiqitin ligase complexes, Cullin-Rbx and Anaphase-promoting complex (APC/C) (annotations from KEGG PATHWAY Database, http://www.genome.jp/kegg/pathway.html). Regulatory protein degradation plays important roles in oocyte development. In Xenopus oocytes, the APC/C was found to degrade XErp1 protein, one component of the cytostatic factor, and this degradation causes the release of the meiosis arrest (Wu and Kornbluth, 2008). APC/C is also involved in the activation of meiotic progression in Drosophila oocytes. A mutation of the *cortex* gene, which encodes a female meiosis specific activator of APC/C, results in the accumulation of Cyclin A protein and blocks meiotic progression (Pesin and Orr-Weaver, 2007). Polyadenylation of *cortex* mRNA occurs during oocyte maturation (Pesin and Orr-Weaver, 2007) and this polyadenylation depends on WISP function (Benoit et al., 2008). My finding suggests that the ubiquitination machinery is probably activated as a consequence of cytoplasmic polyadenylation during late oogenesis.

WISP targets are also associated with the "progesterone-mediated oocyte maturation" pathway (P = 9.4E-4). Out of 29 total components of this pathway detected on the arrays, I identified 25 as WISP regulated. This oocytes maturation pathway is established mainly from Xenopus studies. It has been shown that cytoplasmic polyadenylation plays a central role in activating oocyte maturation in Xenopus (Belloc et al., 2008). Although oocyte maturation in Drosophila is quite different from that in Xenopus, some of the factors in the oocyte maturation pathway, such as cell cycle regulators, could be the same. These factors might play different

roles in Drosophila oocytes but the regulatory function of cytoplasmic polyadenylation in controlling their mRNAs appears to be conserved.

Functional relevance of genes targeted by WISP in early embryos

I also searched for enriched functional groups among the genes that are WISP regulated in early embryos. The 3849 probe sets that were identified as regulated by WISP in early embryo represented 3178 genes. As shown in Table 4.3, enriched functional categories within Biological Process included "enzyme linked receptor protein signaling pathway", "phospholipid metabolic process", "reproductive cellular process". Top-ranking categories in Cellular Compartment suggest that membrane proteins are enriched in WISP targets (*P* values from 3.90E-7 to 9.52E-6). Top categories in Molecular Function are mainly ion binding proteins (*P* values from 1.72E-11 to 1.81E-7).

GO analysis identified a functional group "axis specification" (*P* =3.8E-5). This group includes 83 components that are associated with function in patterning the embryo, such as *bicoid, Toll, torso, caudal, gurken, hunchback, nanos, oskar, pumilio, trunk*. Posttranscriptional regulation of axis patterning genes in Drosophila embryos has been well studied. Some of these genes are already known to be regulated by polyadenylation. For example, the posterior determinant *oskar* mRNA is transported to the posterior pole of the oocyte and translation of the mRNA is repressed during the transport (Kim-Ha et al., 1995; Webster et al., 1997). Translation of *oskar* mRNA in the posterior is activated by cytoplasmic polyadenylation (Castagnetti and Ephrussi, 2003; Chang et al., 1999). Maternally supplied *hunchback* mRNA is another example. *hunchback* mRNA has a short poly(A) tail of about 30 nucleotides in early oocytes, and no *hunchback* translation is detected there (Wreden et al., 1997). Its poly(A) tail elongates to about 70 nucleotides in early embryos; this correlates with the increase of its translation. Although some of these axis formation genes are already known to be

Table 4.3 Over-representation of the gene categories shown by DAVID analysis of *wisp* targets in the 0- to 1-hr embryos.

Term	Count	*P* value
Biological process		
GO:0007167~enzyme linked receptor protein signaling pathway	71	1.38E-07
GO:0006644~phospholipid metabolic process	51	2.44E-06
GO:0048610~reproductive cellular process	192	3.97E-06
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	55	5.60E-06
GO:0007166~cell surface receptor linked signal transduction	150	6.79E-06
GO:0019637~organophosphate metabolic process	54	8.08E-06
GO:0006650~glycerophospholipid metabolic process	37	1.14E-05
GO:0009798~axis specification	83	3.80E-05
GO:0009880~embryonic pattern specification	87	4.62E-05
GO:0046486~glycerolipid metabolic process	40	5.07E-05
GO:0019953~sexual reproduction	245	5.53E-05
GO:0003006~reproductive developmental process	186	6.97E-05
GO:0007276~gamete generation	240	9.42E-05
GO:0030707~ovarian follicle cell development	86	1.12E-04
GO:0009952~anterior/posterior pattern formation	66	1.60E-04
GO:0007292~female gamete generation	197	1.61E-04
GO:0032504~multicellular organism reproduction	244	1.68E-04
GO:0051640~organelle localization	53	1.90E-04
GO:0048477~oogenesis	196	1.92E-04
GO:0030384~phosphoinositide metabolic process	30	2.08E-04
GO:0007350~blastoderm segmentation	79	2.13E-04
GO:0008654~phospholipid biosynthetic process	33	2.26E-04
GO:0051301~cell division	105	2.31E-04
GO:0007424~open tracheal system development	67	2.38E-04
Cellular compartment		
GO:0031224~intrinsic to membrane	347	3.90E-07
GO:0016021~integral to membrane	335	1.56E-06
GO:0005886~plasma membrane	201	9.52E-06
Molecular function		
GO:0008270~zinc ion binding	371	1.72E-11
GO:0046914~transition metal ion binding	426	6.59E-09
GO:0046872~metal ion binding	519	1.90E-08
GO:0043169~cation binding	524	1.81E-07

targets of regulatory polyadenylation, it was not clear if others are also regulated in a poly(A)-dependent way. My finding suggests that cytoplasmic polyadenylation could be a widely-used mechanism that helps to activate these axis formation genes.

Functional relevance of genes regulated by WISP differently in oocytes and embryos

Cytoplasmic polyadenylation of maternal transcripts happens during oogenesis and upon egg activation (Benoit et al., 2008; Cui et al., 2008; Vardy and Orr-Weaver, 2007). It is possible that some maternal transcripts are only polyadenylated at either one of the two stages or at both stages. I looked for probe sets that were detected on both my oocyte and embryo microarrays. A total of 6197 probe sets, which represent 4635 unique genes, were found. I grouped them based on the microarray results (Figure 4.4).

2080 genes are determined as WISP regulated in both the oocyte and the embryo datasets. They could be regulated by cytoplasmic polyadenylation both during oogenesis and egg activation. For example, *cyclin B* mRNA undergoes two rounds of polyadenylation. About 100-120 adenosine residues are added to *cyclin B* mRNA during late oogenesis and then the poly(A) tail further elongates to about 150-200 adenosine residues upon egg activation (Benoit et al., 2005; Vardy and Orr-Weaver, 2007). Transcripts of the 2080 genes could be regulated in the same way as *cyclin B* mRNA. However, I cannot rule out the possibility that they are only polyadenylated in the oocytes, but not at egg activation, because the short poly(A) tails of these mRNAs sustained in *wisp* mutants until after egg activation.

729 genes are determined as WISP targets in the oocytes, but not in the embryos. Poly(A) tail shortening of their transcripts are more severe in *wisp* deficient oocytes (relative to wild type) than in *wisp* deficient embryos. One possibility is that their poly(A) tail is shortened in wild type embryos upon egg activation. From the

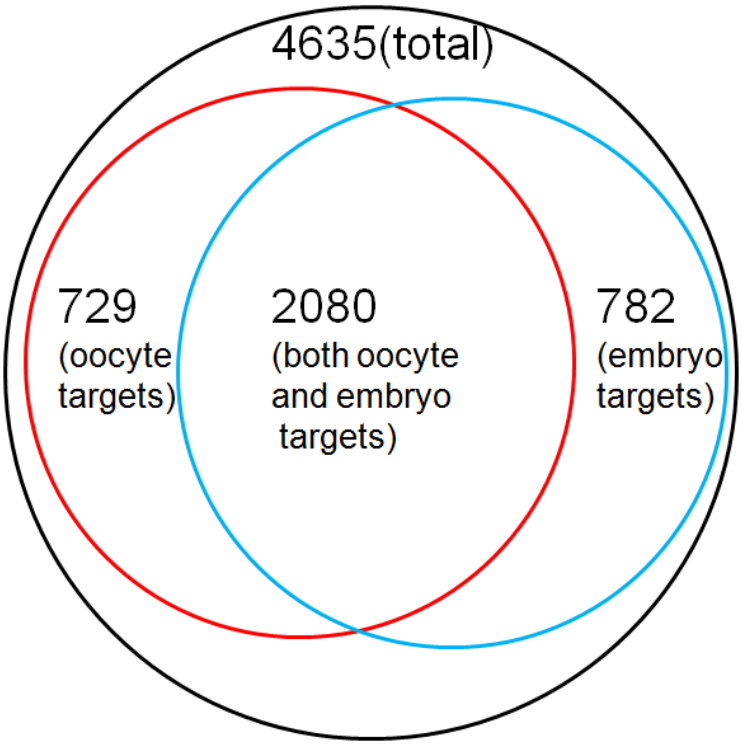


Figure 4.4 Diagram showing overlap of WISP-regulated targets in mature oocytes or early embryos. Among the total 4635 genes, 2080 genes were identified as WISP targets in both oocytes and embryos; 729 targets were identified as WISP targets in oocytes; 782 targets were identified as WISP targets in embryos; 1044 genes were not WISP regulated.

knowledge of poly(A) tail control in Xenopus oocytes, poly(A) tail length of maternal mRNAs is a balance between polyadenylation and deadenylation (Kim and Richter, 2006). Transcripts of these 729 genes are probably only polyadenylated in the oocytes but deadenylated in the embryos in wild type. The oocyte specific polyadenylation suggests that these genes are probably activated during oogenesis and their functions might be related to oocyte development, such as oocyte maturation and female meiosis. Another possibility is that their poly(A) tails are elongated in the *wisp* deficient embryos upon egg activation. Egg activation could activate another poly(A) polymerase to elongate the poly(A) tails. A nuclear PAP, encoded by the *hiiragi* gene, is found in the embryo cytoplasm and it can mediate polyadenylation of maternal mRNAs in the embryos (Juge et al., 2002).

782 genes are only detected as WISP regulated targets in the embryos. They are probably the targets of the cytoplasmic polyadenylation machinery only upon egg activation. These genes might have functions in embryo development, and polyadenylation of mRNAs of these genes upon egg activation could help to activate these mRNAs in the embryo. However, I cannot rule out the possibility that for some of these 782 mRNAs, elongation of their poly(A) tails has already started during oogenesis but the difference in length was not significant enough for detection by the method I used.

Functional relevance of genes not targeted by WISP

1044 genes are determined by the microarray analysis as not regulated by cytoplasmic polyadenylation. It was interesting to test whether any functional group was overrepresented with the mRNAs that are not regulated by WISP. I searched for functional themes among these 1044 genes. Most of the top-ranking GO terms produced by DAVID analysis (listed in Table 4.4) could be grouped into one major class, which contains mRNAs that encode mitochondrial membrane proteins. The

Table 4.4 Over-represented categories of genes that are not regulated by WISP function.

Term	Count	*P* Value
Biological process		
GO:0006091~generation of precursor metabolites and energy	58	1.64E-12
GO:0015980~energy derivation by oxidation of organic compounds	37	5.68E-09
GO:0006119~oxidative phosphorylation	35	2.06E-08
GO:0045333~cellular respiration	31	4.53E-07
GO:0022900~electron transport chain	26	1.54E-06
GO:0042775~mitochondrial ATP synthesis coupled electron transport	22	3.27E-06
GO:0055114~oxidation reduction	76	4.28E-06
GO:0006412~translation	50	4.38E-06
GO:0042773~ATP synthesis coupled electron transport	22	6.15E-06
GO:0022904~respiratory electron transport chain	22	1.11E-05
Cellular compartment		
GO:0005811~lipid particle	64	1.48E-09
GO:0044455~mitochondrial membrane part	34	1.68E-07
GO:0005746~mitochondrial respiratory chain	25	2.68E-07
GO:0070469~respiratory chain	25	5.41E-07
GO:0000502~proteasome complex	23	1.96E-06
GO:0019866~organelle inner membrane	46	3.96E-06
GO:0005743~mitochondrial inner membrane	42	4.08E-06
GO:0044429~mitochondrial part	70	8.26E-06
Molecular function		
GO:0015077~monovalent inorganic cation transmembrane transporter activity	24	1.42E-05
GO:0005198~structural molecule activity	39	1.45E-05

protein products of these genes are part of the oxidative phosphorylation machinery that creates energy in the cell. I found multiple subunits of F-type ATPase, NADH dehydrogenase, Cytochrome c oxidase and Cytochrome c reductase. This suggests that basic cell functions of the oocytes, such as energy production, are probably not regulated by cytoplasmic polyadenylation.

By looking at the pathways that were enriched in this dataset, I found that 23 subunits of the proteasome, out of 29 total detected subunits on the array, are in the non-WISP regulated group. This suggests that mRNAs encoding proteasome components are not regulated by cytoplasmic polyadenylation. Interestingly, in my analysis of the WISP regulated genes in the oocytes, I found that the ubiquitination machinery is regulated by cytoplasmic polyadenylation in late oocytes. Thus clearly the two parts of the ubiquitin-mediated proteolysis pathway are regulated differently at the level of cytoplasmic polyadenylation. These findings suggest that cytoplasmic polyadenylation is a mechanism specific for some maternal mRNAs in the female germline.

Target RNAs can be co-immunoprecipitated with WISP

With such a large part of the maternal transcriptome showing poly(A) tail shortening in *wisp* mutant, the question arises whether these transcripts are direct targets of WISP. Although GLD-2-type poly(A) polymerases do not have RNA binding domains (Wang et al., 2002) and they are believed not to bind RNA directly, there is evidence that GLD-2 proteins can be recruited to the target mRNA by forming complexes with certain RNA binding proteins (Barnard et al., 2004; Kim et al., 2009; Wang et al., 2002). It has also been shown that WISP protein and RNA are in the same ribonucleoprotein (RNP) complex in ovarian extracts (Benoit et al., 2008). It is likely that mRNAs directly regulated by WISP will also be found in such WISP-containing RNP complexes.

I used RNA immunoprecipitation (Keene et al., 2006) to examine if a subset of putative WISP targets were associated with WISP-containing RNPs. Wild type embryos were collected in a 0- to 1-hr collection and fixed using formaldehyde. Cytoplasmic extracts were made from these embryos. WISP containing RNPs were immunoprecipitated from these extracts using an immobilized polyclonal anti-WISP antibody (Cui et al., 2008); pre-immune serum was used in parallel as a negative control. I used real-time quantitative PCR to examine the enrichment of RNA that was immunoprecipitated with WISP. Test mRNAs included seven known targets of WISP (Benoit et al., 2008; Cui et al., 2008) and three targets identified in this microarray study and confirmed with PAT assays in Figure 4.3C. As shown in Figure 4.5, all of the ten test mRNAs were enriched by > 2-fold in the WISP IP compared with the mock IP. But neither of the two negative control mRNAs, *rpA1* and *CG3083*, were significantly enriched in WISP IP. This suggests that the ten WISP targeted mRNAs and WISP protein are co-present in the same RNPs. These ten mRNAs are likely to be directly regulated by WISP.

Conclusions

My microarray analysis shows that poly(A) tail elongation of thousands of maternal mRNAs depends on the function of the WISP protein. Thus, cytoplasmic polyadenylation is a widely used mechanism that regulates maternal mRNAs in oocyte development and early embryogenesis.

Previous studies have shown that specific mRNAs encoding axis patterning proteins, such as *oskar* (Castagnetti and Ephrussi, 2003; Chang et al., 1999) and *bicoid* (Salles et al., 1994), or cell cycle regulators, such as *cyclin A* (Vardy et al., 2009) and *cyclin B* (Benoit et al., 2005; Vardy and Orr-Weaver, 2007) undergo poly(A) tail elongation during oocyte development or egg activation. Elongation of the poly(A) tails is concomitant with activation of translation of these mRNAs. My microarray

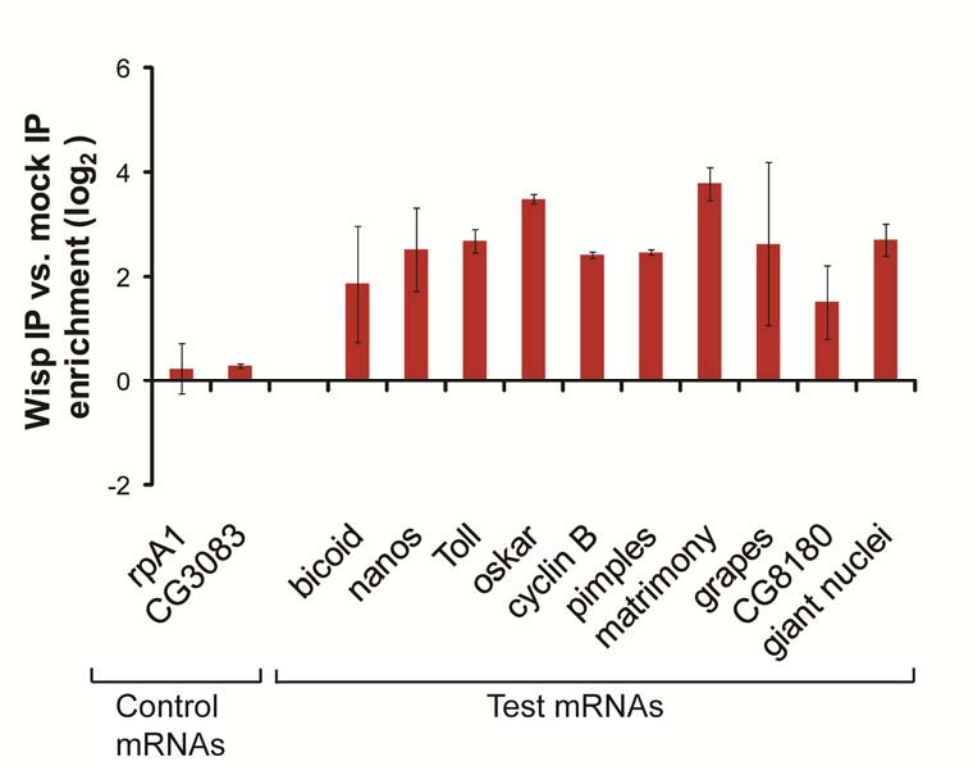


Figure 4.5 RNA immunoprecipitation. Embryos collected at 0- to 1-hr post deposition from wild type flies were fixed and cross-linked in formaldehyde. Cytoplasmic extracts from these embryos were incubated with an immobilized polyclonal anti-WISP antibody or pre-immune serum (negative control). mRNAs enriched in the IPs were isolated and used to synthesize cDNA. RT-PCR was performed to test enrichment of mRNAs in the WISP IP vs. control IP. All data were normalized to *RpL32*. The average of two biological replicates is shown. Error bars indicate range of errors.

data indicate that axis patterning genes and cell cycle regulators are indeed the two groups most highly represented in the genes that are under cytoplasmic poly(A) tail regulation.

Although it appears that a large part of the maternal transcriptome undergoes cytoplasmic polyadenylation, there is still target specificity in this regulatory mechanism. My microarray data showed that in the protein degradation pathway, only mRNAs encoding the ubiquitination machinery are under the control of cytoplasmic polyadenylation, but mRNAs encoding subunits of the proteasome are not regulated. This suggests that not all of the maternal mRNAs are regulated by WISP.

My findings will help to understand the mechanism of WISP-dependent cytoplasmic polyadenylation during oogenesis and upon egg activation. Future studies on the genes whose poly(A) tail length is regulated by WISP will help to further understand how these genes function in oogenesis or egg activation.

CHAPTER 5

CG2941 IS A NOVEL GENE NECESSARY FOR DROSOPHILA FEMALE FERTILITY

5.1 Introduction

Fertilization results from the fusion of gametes. One feature which makes Drosophila fertilization different from mammalian fertilization is that Drosophila produce giant sperm, which can be about 300 times longer than human sperm (Pitnick et al., 1995). Drosophila sperm are stored in the female sperm storage organs after mating and can be used to fertilize the egg for weeks after storage (Neubaum and Wolfner, 1999).

In Drosophila, after an initial arrest in prophase I during oocyte development, meiosis of fully grown oocytes arrests at metaphase I (Foe et al., 1993). Ovulation then triggers egg activation, which releases the egg meiosis I arrest, permitting completion of meiosis (Heifetz et al., 2001). Sperm enters the egg during female meiosis. In wild-type Drosophila eggs, after both meiotic divisions are completed, the four meiotic products decondense their chromosomes (Foe et al., 1993). If the egg is not fertilized, all four nuclei re-condense their chromosomes and come together at the egg periphery and arrest in a metaphase-like state. In fertilized eggs, the sperm nucleus reorganizes to become a male pronucleus with maternally deposited nuclear proteins (Liu et al., 1997). The most centrally located female meiotic product becomes the female pronucleus and migrates toward the male pronucleus; the other three haploid meiotic products condense their DNA and move to the periphery, become polar bodies that do not participate in further development. When the pronuclei are closely apposed, the male-provided centrosome divides and assembles the first zygotic spindle (Callaini

and Riparbelli, 1996). Paternal and maternal chromosomes align on separate sides of the first mitotic spindle. During anaphase they migrate toward the spindle poles (Foe et al., 1993). The first two zygotic nuclei, which form at the end of the first mitotic division then undergo rapid nuclear divisions without cytokinesis to form the syncytial blastoderm.

Initiation of embryogenesis is under the control of developmental cues from both parents. Both paternal and maternal effect genes have been identified to be required for fertilization or the subsequent nuclei division in the embryo. Among male contributions, for example, *ms(3)sneaky (snky)* encodes an acrosomal membrane protein and is required for sperm membrane breakdown (Wilson et al., 2006). Another paternal gene, Drosophila ferlin gene *misfire (mfr)*, is required for sperm head decondensation in the egg (Smith and Wakimoto, 2007). The paternal effect mutation *ms(3)K81(K81)* prevents the incorporation of the paternal chromosomes into the zygote nucleus (Loppin et al., 2005b; Yasuda et al., 1995) and the maternal effect mutation *maternal haploid (mh)* results in a similar phenotype (Loppin et al., 2001). Maternal effect genes are also important in the first zygotic division. For example, *sesame (ssm)* encodes the histone chaperone HIRA and is necessary for the formation of the male pronucleus (Loppin et al., 2005a). KLP3A, which encodes a kinesin-like protein, affects the migration of the female pronucleus (Williams et al., 1997). And the *Young arrest (Ya)* gene is needed for the pronuclei to enter the first mitotic division (Sackton et al., 2009).

In Chapter 4, I found that many maternal mRNAs undergo poly(A) tail elongation during late oogenesis and early embryo development. This poly(A) tail elongation appears to be related to the developmental functions of these transcripts. For example, *cyclin A* mRNA is polyadenylated and translated during oocyte maturation and Cyclin A level is important for meiotic progression (Vardy et al.,

2009). Studying the function of the transcripts that are regulated by WISP could help to further understand oogenesis and embryo development. To identify new genes that are involved in female germline development, I screened for new female sterile mutations in a collection of potential mutant lines of WISP targeted genes. Here I show one example, the CG2941 gene. I found it encodes a novel protein that is needed for female fertility. Eggs produced by CG2941 mutant females arrest very early in embryo development. Fertilization does not occur normally in eggs in the absence of CG2941 function.

5.2 Materials and Methods

Drosophila stocks and crosses: Fly strains of *CG2941*^{KG08206}/FM7c, *Df(1)cho2*/FM7c, Δ2-3 P transposase and *tubulin*-GAL4/TM3, *Sb* were obtained from the Bloomington Drosophila Stock Center (Indiana). I crossed virgin females of *Df(1)cho2*/FM7c deficiency strain to *CG2941*^{KG08206} males to generate hemizygous daughters. To generate P-element excision lines, virgin female flies of *CG2941*^{KG08206}/FM7c were crossed to Δ2-3 males which express the P transposase. Progeny were crossed to FM7c flies to score for the loss of P-element using genomic PCR. RNAi lines carrying UAS-driven snapback RNA against CG2941 were obtained from the Vienna Drosophila RNAi Center (stock#52356, Vienna, Austria).

RNA extraction, quantitative PCR and PAT assays: Total RNA was extracted from dissected ovarian oocytes or 0- to 1-hr embryos and used to synthesize cDNA as described in Chapter 2. qPCR was then performed to determine the abundance of CG2941 and CG2938 transcripts in the extract. Results were normalized to the abundance of RpL32 (Cui et al., 2008). PAT assays (Salles et al., 1994) were used to assess the length of poly(A) tail on CG2941 transcript. Sequences of primer used were

CG2941 upper 5’ ATGCCCTACGCTGGGACTTG

CG2941 lower 5’ CGCTAAGATCGCCCATAACAG

CG2938 upper 5’ CTTCTGGTCCTCGGCTTCATC

CG2938 lower 5’ GAAGGCGAGGTAACGCAAACA

CG2941 PAT 5’ TTGGCAAGCAAGTGAAGTTATT

Western blot analysis: Samples from ovarian oocytes were prepared for Western blotting as described in Chapter 2. A polyclonal anti-CG2941 antibody was generated using the carboxy-terminal 272 amino acids of the predicted CG2941 protein as antigen. Antibody was affinity purified with the GST-CG2941 fusion protein. The purified anti-CG2941 antibody was used at 1:100 dilution to probe Western blots.

Embryo collection, fixation and staining: As previously described in Chapter 3, embryos were collected at 0- to 2-hr post egg deposition produced from 3- to 4-day-old virgin homozygous *CG2941*^{KG08206} females that had been mated with wild-type Oregon-R P2 males. Fixation and staining was performed using the method described in Chapter 3. A mouse anti-α-tubulin antibody was used at 1:200 dilution to visualize microtubule structure and propidium iodide was used to visualize DNA. An anti-sperm tail antibody (kindly provided by T. Karr) (Karr, 1991) was used at 1:1000 dilution to examine fertilization.

5.3 Results and Discussion

Disruption in the CG2941 gene locus causes a female fertility defect: To look for new mutations that cause female fertility defects, I screened 45 potential mutant lines of genes (listed in Table 5.1) whose poly(A) tails are significantly shortened in the *wisp* mutant, as predicted by my microarray study in Chapter 4. I obtained potential gene disruption lines created by the Berkeley Drosophila Genome

Table 5.1 List of fly lines tested for female fertility.

Bloomington No.	Genotype
10144	w[1118] P{w[+mC]=EP}Pk17E[EP438] CG6961[EP438]
12166	w[1118]; P{w[+mC]=lacW}nero[s1921]/TM3, Sb[1]
12825	w[1118] P{w[+mGT]=GT1}CG12994[BG02800]
12965	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG00869 ry[506]
13548	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Edem2[KG00637]
13648	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Pep[KG00294] ry[506]/TM3, Sb[1] Ser[1]
13731	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}whd[KG01596]
13902	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Fit1[KG05576] ry[506]
14121	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Tina-1[KG05573]
14324	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}CG12006[KG07161] ry[506]
14431	y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}CG30118[KG03769]/CyO; ry[506]
14847	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG16971[EY00429] CG33229[EY00429]
14942	y[1] P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG08206/FM7c
15110	y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}olf186-F[KG07396]/CyO; ry[506]
15194	y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG09529; ry[506]
15743	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG1553[EY04377] sax[EY04377]
15879	y[1] w[67c23] P{w[+mC] y[+mDint2]=EPgy2}CG32732[EY02714]
15981	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG18870[EY06926]
15983	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG5728[EY06964]
15999	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}whd[EY07800]
17014	w[1118] P{w[+mC]=EP}CG6961[EP1607]
17059	w[1118]; P{w[+mC]=EP}nrv1[EP2454]
17090	w[1118]; P{w[+mC]=EP}RhoBTB[EP3099]/TM3, Sb[1]
17123	w[1118]; P{w[+mC]=EP}dsd[EP3400]/TM6B, Tb[+]
17338	y[1] w[67c23] P{w[+mC] y[+mDint2]=EPgy2}CG18259[EY04931]
17608	y[1] P{w[+mC] y[+mDint2]=EPgy2}CG14805[EY09810] w[67c23]
18212	w[1118]; PBac{w[+mC]=RB}CG11180[e03938]/CyO
18818	w[1118]; PBac{w[+mC]=WH}CG5508[f04927]/TM6B, Tb[1]
20128	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG30118[EY01823]/CyO

Table 5.1 (Continued)

Bloomington No.	Genotype
20456	y[1] w[67c23]; P{y[+t7.7] w[+mC]=wHy}CG16971[DG23208] CG33229[DG23208]
21030	y[1] w[67c23]; P{y[+t7.7] w[+mC]=wHy}Edem2[DG03809]
21030	y[1] w[67c23]; P{y[+t7.7] w[+mC]=wHy}Edem2[DG03809]
21527	y[1] w[67c23]; P{y[+t7.7] w[+mC]=wHy}CG31457[DG16304]
22114	w[1118]; P{w[+mC]=EPg}CG14516[HP31652]/TM3, Ser[1]
22153	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG14764[EY19265] CG34430[EY19265]
22297	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}CG5508[EY18945]
22393	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}Pect[EY20477]
22411	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}whd[EY20655]/CyO
23253	y[1] w[*]; P{w[+mC]=UAST-YFP.RabX6.S22N}Myo31DF[01]
23273	y[1] w[*]; P{w[+mC]=UASp-YFP.Rab9.Q71L}10
23971	y[1] w[67c23]; Mi{ET1}CG18347[MB02413]
24270	w[1118]; Mi{ET1}CG18812[MB03763]
25294	w[1118]; Mi{ET1}CG4751[MB05404]
26065	w[1118]; Mi{ET1}RhoBTB[MB07044]
26066	w[1118]; Mi{ET1}CG14516[MB07062]
26363	w[1118]; Mi{ET1}Pi3K68D[MB08286] CG14131[MB08286]

Project that had a P-element insertion in or near the gene region of interest. Homozygous virgin female flies were collected from each line and crossed to wild-type Oregon-R P2 males to test for fertility.

One line, *CG2941*^{KG08206}, showed a defect in female fertility (Table 5.2). Only 16.9% of the 516 eggs collected from homozygous *CG2941*^{KG0820} females hatched into progeny. Hemizygous females carrying the *CG2941*^{KG0820} chromosome over a deficiency that covers the CG2941 locus also showed a reduced hatchability rate of their progeny (14.5% of 249 eggs). Heterozygous *CG2941*^{KG0820}/+ females used as control flies produced 544 eggs and 95.8% of these eggs hatched. This indicates that the *CG2941*^{KG0820} line has a defect in producing eggs that can development into viable animals. To confirm further that the defect is caused by the P-element insertion, I generated two independent excision lines by crossing the original P-element insertion line to a fly line that expresses the Δ2-3 P transposase to induce the excision of the P-element from its original insertion site. In both lines, the hatchability rate of embryos laid by females homozygous of the excision chromosome increased to 98.9% and 98.7%, respectively. This result indicates that the hatchability defect in the original line is due to the P-element insertion.

Maternal CG2941 transcripts are regulated by cytoplasmic polyadenylation: My microarray study in Chapter 4 identified CG2941 as a WISP regulated target. To confirm if CG2941 is regulated by cytoplasmic polyadenylation, I used the PAT assay to examine the poly(A) tail length of CG2941 mRNA in the absence of WISP, which is the major poly(A) polymerase in developing eggs. As shown in Figure 5.1, in stage 14 oocytes from *wisp*^{41}*/Df(1)RA47* mutant females, CG2941 poly(A) tail length showed a slight decrease compared to ~50 nt in the normal oocytes from *wisp*^{41}/+ control females. Upon egg activation, CG2941 poly(A) tail length increased to ~60 nt in the control sample. However in wisp deficient

Table 5.2 Hatchability of CG2941 mutant flies. 3-days old virgin females of the genotypes indicated in the table were crossed to wild-type males. Hatchability was calculated for each genotype as number of progeny / number of egg.

Genotype	Hatchability	No. of eggs (n)
CG2941^{KG08206}/FM7c	95.8%	544
CG2941^{KG08206}/ CG2941^{KG08206}	16.9%	516
CG2941^{KG08206}/ Df	14.5%	249
Excision line 1	98.9%	883
Excision line 2	98.7%	1206

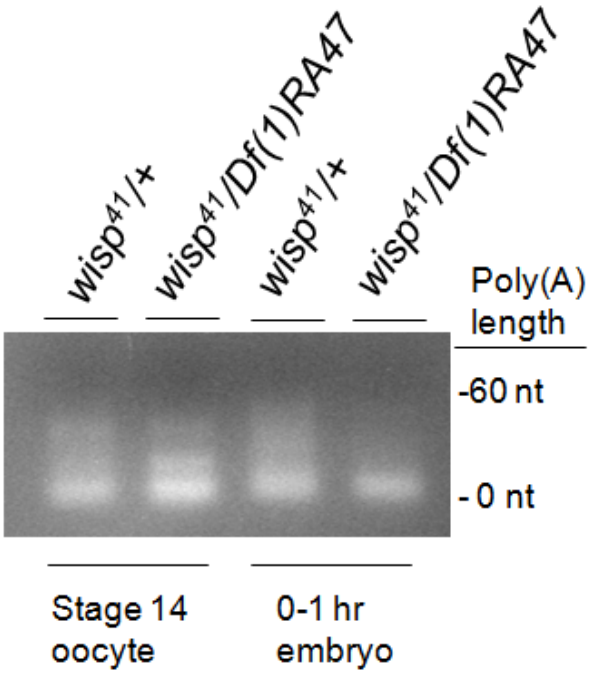


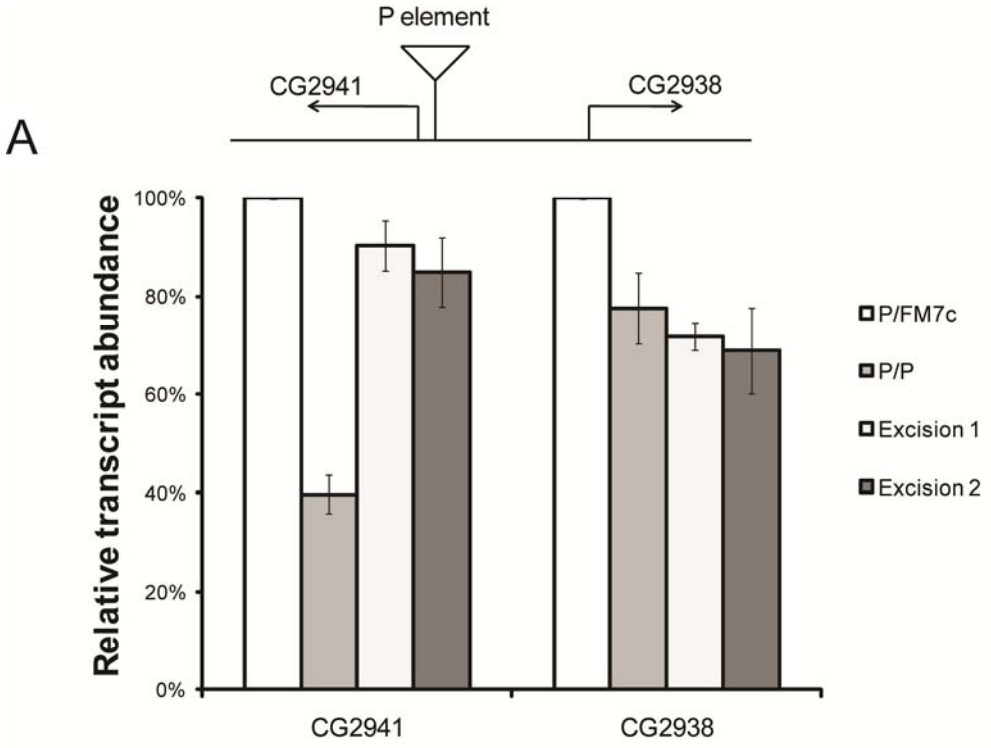
Figure 5.1 PAT assays for CG2941. RNA was extracted from stage 14 oocytes or 0- to 1-hr embryo produced by *wisp*^{41}/+ control and *wisp*^{41}*/Df(1)RA47* mutant females. PAT assays were performed to examine the poly(A) tail length of *CG2941* mRNA. Molecular size markers are shown on the right.

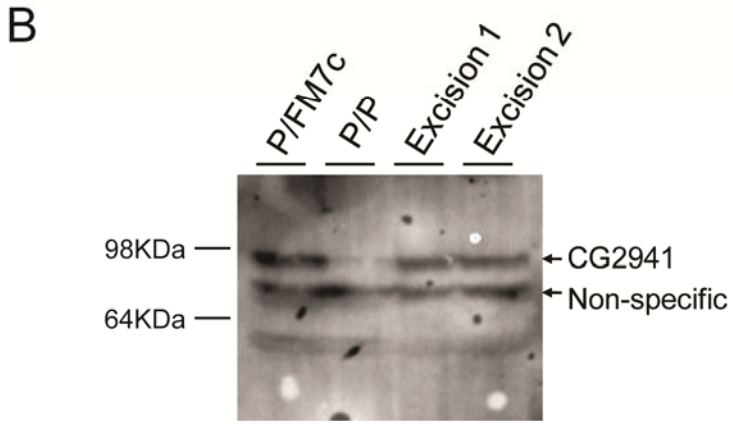
embryos, CG2941 poly(A) tail length decreased to ~10 nt. These results indicated that the poly(A) tail of CG2941 transcripts is regulated by WISP in the oocytes and early embryos.

CG2941 encodes a novel conserved protein: According to the latest annotation of the Drosophila genome (http://www.flybase.org), CG2941 has one predicted open reading frame (ORF) that encodes a protein of 911 amino acids. BLAST searches revealed that the predicted CG2941 protein is conserved in all twelve Drosophila species whose genomes have been sequenced. Similarities among these CG2941 homologs are from 57% to 94%. No CG2941 homologs are found in other animal species, implying that CG2941 could play a role specific to Drosophila. CG2941 protein does not contain any motifs with known functions.

CG2941 RNA and protein levels are decreased in ovarian oocytes from homozygous *CG2941^{KG08206}* females: The P-element in *CG2941^{KG08206}* is inserted into the CG2941 locus at 21 base pairs upstream of the predicted transcription start site and thus is in the putative regulatory region of the gene. I used real-time quantitative PCR to examine the abundance of CG2941 transcripts in ovarian extracts. The transcript level of CG2941 in ovaries of homozygous *CG2941^{KG08206}* females decreased to about 40% of the wild-type level (Figure 5.2A). Excision of the P-element increased the CG2941 transcript level to about 80-90% relative to wild-type. The transcript level of a neighbor gene CG2938 in the homozygous P-insertion line slightly decreased to about 80% of wild-type level but was about the same as the CG2938 transcript levels in the two excision lines. The difference of CG2938 transcription could be due to the different genetic background between control and P-element lines. This result suggests that the P-element insertion significantly disrupts the transcription of the CG2941 gene.

Figure 5.2 The expression levels of CG2941 in ovarian oocytes. (A) Transcript abundance by qPCR. Total RNA was extracted from ovarian oocytes dissected from females of the genotypes indicated in the figure. qPCR was used to examine the abundance of transcript (normalized to *RpL32*). Results are shown in percentage relative to the wild-type level. Error bars show range of error of two biological replicates. (B) Protein level by Western blot analysis. Total protein extracts from ovarian oocytes dissected from females of the genotypes indicated in the figure were separate by SDS-PAGE and probed with anti-CG2941 antibody. The 85KDa band is a non-specific band based on the predicted size of CG2941 protein (95KDa). "P" in the figures stands for the P-element insertion $CG2941^{KG08206}$.


A
P element
CG2941
CG2938
Relative transcript abundance
100%
80%
60%
40%
20%
0%
CG2941
CG2938
P/FM7c
P/P
Excision 1
Excision 2


B
P/FM7c
P/P
Excision 1
Excision 2
98KDa
64KDa
CG2941
Non-specific

I generated an antibody against CG2941 and used western blot analysis to test the amount of CG2941 protein in ovarian oocytes made from wild-type female or homozygous *CG2941*^{KG08206} females. As shown in Figure 5.2B, my antibody detects a protein of ~95 KDa, as predicted, in ovarian protein extracts of wild-type and the two excision lines. The protein level of CG2941 was significantly decreased in the ovarian protein extracts of homozygous *CG2941*^{KG08206} females.

A decreased amount of the transcript and protein of CG2941 was detected in homozygous *CG2941*^{KG08206} flies. This suggests that the *CG2941*^{KG0820} allele is not a biochemically null mutant.

More than half of the eggs produced by homozygous *CG2941*^{KG08206} females arrest very early in embryogenesis: Homozygous *CG2941*^{KG08206} females can produce eggs, but a large proportion of these eggs do not hatch. This result indicates a developmental arrest very early in embryogenesis. To determine the arrest point, I examined several aspects of early embryo development. To test if arrest was due to abnormal egg activation, I examined vitelline membrane. Modifications to the egg coverings are part of egg activation in many species. The vitelline membrane, one of Drosophila's egg coverings, is permeable to small molecules before activation, but after activation vitelline membrane proteins become crosslinked to render the activated egg impermeable to small molecules (Heifetz et al., 2001; LeMosy and Hashimoto, 2000; Mahowald et al., 1983). Activated eggs are consequently impermeable to bleach and remain resistant to lysis after a 2-min incubation in 50% bleach, while ~100% of eggs that have not yet been activated are lysed by this treatment (Mahowald et al., 1983).

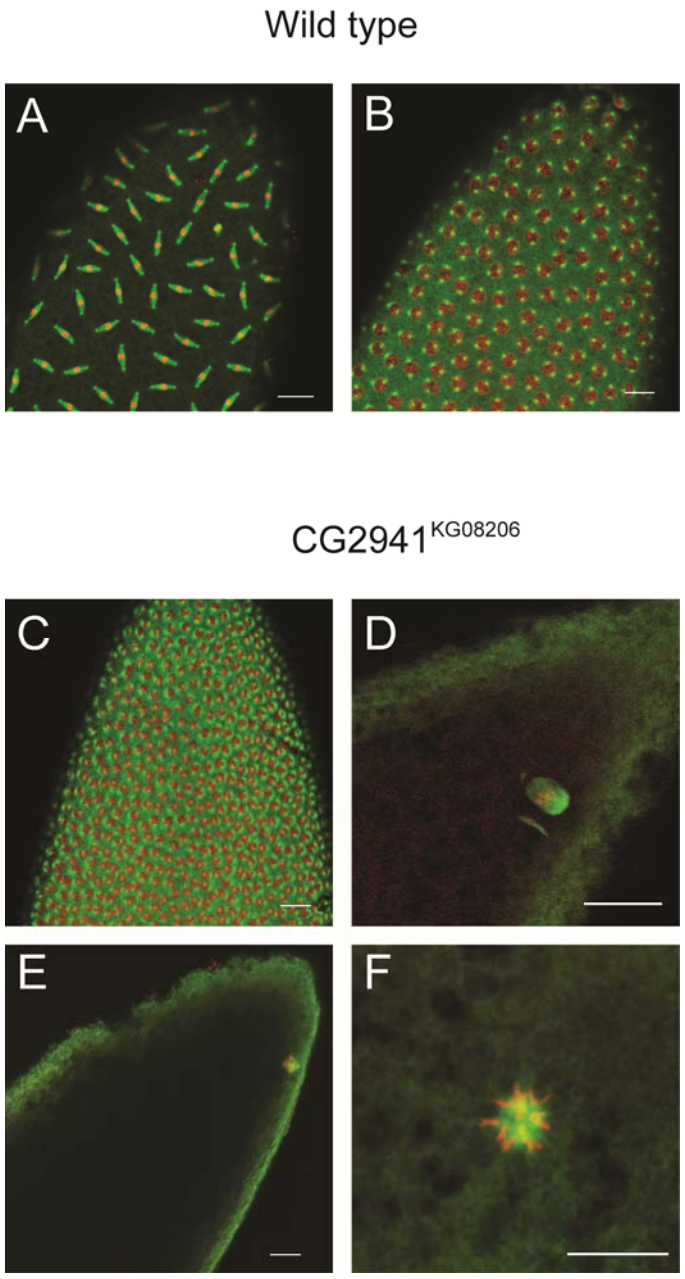
I assayed resistance to 50% bleach to determine if the eggs laid by homozygous *CG2941*^{KG08206} females are defective in egg activation. Zero- to 1-hr eggs laid by either *CG2941*^{KG08206}/+ (control) or homozygous *CG2941*^{KG08206} females were

collected and incubated in 50% bleach for 2 minutes. In one representative experiment, 96.8% of *CG2941*^{KG08206}/+ (N=562) eggs were resistant to bleach incubation, while 96.3% of the eggs (N=489) laid by homozygous *CG2941*^{KG082061} females were resistant to bleach. These data indicate vitelline membrane crosslinking occurs normally in eggs produced by *CG2941* mutant females, suggesting that egg activation is probably normal in *CG2941* deficient embryos. I did not test other aspects of egg activation and it remains possible that there is a defect in egg activation.

I next examined at what stage the developmental arrest occurs in these eggs. In wild type embryos, female meiosis completes upon egg activation and four haploid meiotic products form (Foe et al., 1993). If the egg is fertilized, the sperm nucleus remodels (in part by exchanging protamines for histones) and becomes the male pronucleus. One of the four female meiotic products migrates toward, and apposes with, the male pronucleus, and then the two apposed pronuclei together enter the first mitotic division followed by rapid nuclei divisions. The other three female meiotic products stay at the cortex of the embryo and form a polar body rosette. Wild-type Drosophila eggs can activate without fertilization. Such unfertilized eggs can complete female meiosis but do not develop further (Doane, 1960; Foe et al., 1993).

I collected 0- to 2-hr laid eggs from 3-days old heterozygous *CG2941*^{KG08206}/+ females (control) and homozygous *CG2941*^{KG08206} females that had been mated to wild type OR-P2 males. Eggs were stained with anti-α-tubulin to visualize microtubule structure and with propidium iodide to visualize DNA. As expected, the majority of 0- to 2-hr embryos laid by heterozygous control females were undergoing embryonic mitotic divisions (Figures 5.3A and 5.3B). About 44% of the eggs (n=108) laid by homozygous *CG2941*^{KG08206} females undergo normal embryonic mitosis (Figure 5.3C), which might be due to the residual level of CG2941 protein. The other 56% of the eggs were arrested with very few nuclei or no nuclei in the cytoplasm.

Figure 5.3 Many eggs laid by homozygous *CG2941*^{KG08206} females arrest early in embryogenesis. 0- to 2-hr eggs were collected from heterozygous *CG2941*^{KG08206}/+ females or homozygous *CG2941*^{KG08206} females that had been mated to wild type males. Fixed eggs were stained with propidium iodide to visualize DNA (red) and with anti-α-tubulin antibody to visualize microtubule (green). 100% (n=97) of control eggs undergo embryonic mitosis (A and B). Normal mitosis was only observed in about 44% (n=108) of the eggs from mutant mothers (C). Occasionally (3%) a giant spindle was formed (D). 33% of the mutant embryos appear unfertilized with a normal looking polar rosette (E and F). In the remaining 20% of the eggs, no chromosome or spindle structure was observed. Bar, 10 μm.


Wild type
A
B
CG2941KG08206
C
D
E
F

Occasionally (3%) a giant spindle was formed (Figure 5.3D). About half of the arrested eggs display a normal looking polar body rosette (Figures 5.3E and 5.3F), which is similar to the unfertilized eggs. In the remaining 20% of the eggs, no chromosome or spindle structure was observed. Chromosomes in these eggs could be dispersed or degraded. This result indicates that the arrest of *CG2941*^{KG08206}eggs is very early in embryogenesis.

During Drosophila fertilization the entire sperm enters the egg; the sperm tail can be seen in the cytoplasm of the embryo (Snook and Karr, 1998). To test whether or not eggs from homozygous *CG2941*^{KG08206} females are fertilized, I collected 0- to 30-min eggs from these females and stained them with an anti-sperm tail antibody. Sperm tail staining was found in about 41% of the eggs (n=44). I next used anti-sperm tail antibody and propidium iodide to double-label sperm tail and DNA in the same egg. All the eggs with sperm tail staining (n=18) also had multiple nuclei (>8) in the cytoplasm (Figure 5.4), indicating these eggs were in mitosis. I never observed sperm tail staining in the eggs arrested with the polar rosette (n=11). These results suggest that lack of fertilization is the reason that leads to the lack of development in a proportion of the eggs produced by homozygous *CG2941*^{KG08206} females. I conclude that CG2941 function is needed for fertilization.

CG2941 gene is required for viability: To create a null allele of CG2941, I induced imprecise P-element excision by expressing the Δ2-3 transposase in the flies carrying the *CG2941*^{KG08206} chromosome. By screening about 200 fly lines using PCR to amply the CG2941 gene region, I found two lines with a deletion in the CG2941 gene due to translocation of the P-element. No homozygous flies were produced for either deletion, indicating that an essential gene, potentially CG2941, was disrupted on the chromosome carrying the deletion. I cannot further link the lethality of these two lines to the CG2941 locus using genetic approaches because CG2941 gene is on the X

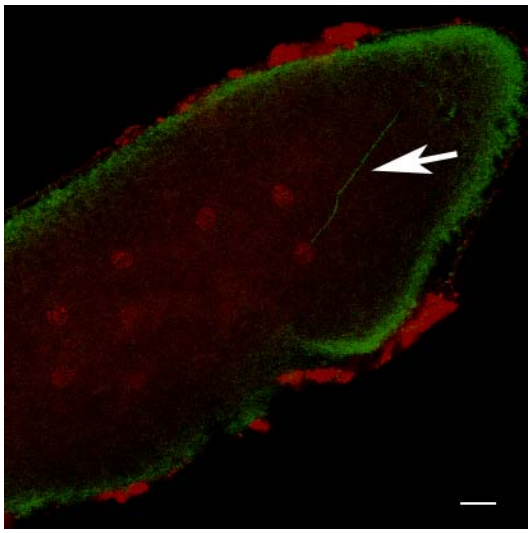


Figure 5.4 Sperm tail can only be seen in a proportion of the eggs produced by homozygous *CG2941*^{KG08206} females. Eggs collected at 0- to 30-min after deposition by homozygous *CG2941*^{KG08206} females were fixed and stained with propidium iodide to visualize DNA (red) an anti-sperm tail antibody (1:1000 dilution), a kind gift from T. Karr (University of Bath, UK) (Karr 1991) to visualize sperm tail (green). A sperm tail (arrow) can be found in eggs undergoing mitosis (n=18) (in figure). No sperm tail staining was observed in the eggs arrested with the polar rosette (n=11). Bar, 10 μm.

chromosome. I cannot rule out the possibility that the lethality is caused by the re-insertion of the excised P element into a second gene locus that is essential for fly viability.

To further examine if the CG2941 gene is essential for viability, I used RNAi to knock down CG2941 in flies. Flies (VDRC stock#52356) carrying a UAS-construct that drives snapback RNA for CG2941 were crossed to flies carrying the *tubulin*-GAL4 driver (Avila and Wolfner, 2009). The progeny should have ubiquitous expression of the RNAi construct in the whole fly throughout development. Only non –RNAi control (Stubble) but no CG2941 knockdown (non-Stubble) progeny can be produced from this cross, indicating function of the CG2941gene is required for viability.

Summary: I have identified a new gene CG2941 whose function is needed for female fertility in Drosophila. A P-element which was inserted in the regulatory region of CG2941 resulted in a decrease of both transcript and protein levels of the gene product in the ovarian oocytes. Reduction of CG2941 expression in the eggs led to a very early arrest in embryogenesis. It is probably because these eggs were not fertilized in the absence of CG2941 function.

CHAPTER 6

DISCUSSION

WISP, a Drosophila GLD-2 homolog, is involved in cytoplasmic polyadenylation in the female germline

Studies in several organisms suggest that maternal mRNAs are tightly regulated by cytoplasmic polyadenylation (Huarte et al., 1992; Kim and Richter, 2006). Cis-elements in the 3'UTR sequence of the mRNA together with a group of protein factors determine when and where the particular mRNA can be polyadenylated (Richter, 2007). Recent studies in Xenopus and *C. elegans* have shown that cytoplasmic polyadenylation is mediated by a special type of PAP, GLD-2 famliy proteins, which are important for the regulation of maternal mRNAs (Barnard et al., 2004; Wang et al., 2002)

Posttranscriptional regulation of maternal mRNAs plays a central role in Drosophila early embryogenesis. In many cases, regulation of mRNA translation involves controlling the poly(A) tail. However, the mechanism of this poly(A) tail control is not well understood in this system. My study demonstrated that the cytoplasmic specific poly(A) polymerase GLD-2, is also conserved in Drosophila. I identified the two GLD-2 proteins in the Drosophila genome. One GLD-2 which is encoded by the *wisp* gene is specifically expressed in the female germ-line cells. Mutations in this gene are associated with female fertility defects. The other GLD-2 is encoded by the CG5732 gene, which is expressed in the testis of the male flies. Males lacking this gene cannot complete spermatogenesis.

In this dissertation, I focused on WISP, the female specific one, and investigated its role in cytoplasmic polyadenylation of maternal transcripts in the

Drosophila female germline. Female meiosis is aberrant in the absence of WISP function and *wisp* deficient embryos arrest very early in development. I demonstrated that lack of WISP directly affects poly(A) tail elongation of maternal mRNAs. My analysis suggests that poly(A) tail elongation is required to activate maternal transcripts during oocyte maturation. Poly(A) tail elongation of *dmos* mRNA does not occur during oocyte maturation in *wisp* deficient oocytes. The MAPK cascade is not activated in these oocytes. WISP is also required for polyadenylation during egg activation. Poly(A) tail elongation of *bicoid*, *Toll* and *torso* mRNAs depends on WISP function.

I used microarray analysis to identify systematically maternal transcripts that are regulated by cytoplasmic polyadenylation. I found many maternal transcripts are regulated by WISP. Activation of polyadenylation appears to be related to the developmental functions of these transcripts. Studying the function of these transcripts could help to further understand how oogenesis and embryo development are regulated.

In Chapter 5, I showed one example, the CG2941 gene. I found it encodes a novel protein that is needed for female fertility. Eggs produced by CG2941 mutant females arrest very early in embryo development. Fertilization does not occur normally in these eggs. Future study on the function of the CG2941 protein will help to understand how it is involved in egg development. Because CG2941 is required for both female fertility and viability, making germline clones with mutations in the CG2941 gene could help to dissect its maternal function from it somatic function. Examining the subcellular localization of the CG2941 protein in oocytes and early embryos could also help to explain why its function is required during fertilization. Because CG2941 is a novel protein, identifying its binding proteins might provide a hint of how it functions.

Poly(A) regulation of maternal mRNAs in Drosophila female germline

In Drosophila oocytes, many maternal mRNAs are found to have a very short poly(A) tail in the early stages during oogenesis, such as *cyclin B* (Benoit et al., 2005; Pesin and Orr-Weaver, 2007; Vardy and Orr-Weaver, 2007), *oskar* (Benoit et al., 2005), *cortex (Benoit et al., 2008; Pesin and Orr-Weaver, 2007)*, *bicaudal-C* (Chicoine et al., 2007), *cyclin A* (Vardy et al., 2009) and *dmos* (Cui et al., 2008). It is probably the same case in Drosophila as in vertebrates that maternal mRNAs are transcribed with long poly(A) tails and then depolyadenylated in the cytoplasm so that they are kept as translational inactive during early oocyte development.

It is known from Xenopus studies that poly(A) regulation during oogenesis is actually determined by the balance between polyadenylation and deadenylation (Kim and Richter, 2006). Early in oogenesis, the PARN deadenylase is recruited to maternal mRNAs and thus these mRNAs are kept in a dormant state with short poly(A) tails. When the oocyte matures, deadenylase activity is repressed and thus poly(A) polymerase can elongate poly(A) tails on specific mRNAs, which leads to translational activation of these mRNAs. Release of PARN is due to a phosphorylation modification on CPEB by a kinase downstream of the hormonal signaling pathway that triggers oocyte maturation (Mendez et al., 2000b; Sarkissian et al., 2004).

The adenylation and deadenylation balance model appears to be present in Drosophila as well. Although PARN has not been found in Drosophila so far, studies on maternal mRNAs suggest a similar mechanism in poly(A) tail length regulation. The deadenylase CCR4, encoded by the *twin* gene, is involved in regulating *cyclin A* and *cyclin B* mRNA poly(A) tails in oogenesis (Morris et al., 2005; Zaessinger et al., 2006). My finding that WISP is the major poly(A) polymerase involved in poly(A) elongation during oogenesis fits the polyadenylation-deadenylation equilibrium model. Poly(A) tail length of maternal mRNAs in Drosophila oocytes is likely the outcome of

the balance of CCR4 and WISP activities. Bic-C is likely to be involved in determining the balance, because Bic-C regulates poly(A) tail in both directions during oogenesis, deadenylation early and then polyadenylation later on (Chicoine et al., 2007). Early in oogenesis, CCR4 could be more active and shifts the balance, which leads to the deadenylation of maternal mRNAs. Later on in oogenesis the balance is switched to the WISP side which results in elongation of mRNA poly(A) tails. It has been shown that Bic-C can interact with WISP (Benoit et al., 2008; Cui et al., 2008). Therefore it is likely that Bic-C and WISP works together as a complex to promote poly(A) elongation during oogenesis. Bic-C is the switch of deadenylation and polyadenylation. It will be interesting to know how this switch happens and if other genes are needed to trigger the switch.

Poly(A) tail elongation occurs during late oogenesis and also during egg activation. My data suggest that WISP is a major poly(A) polymerase used at both times. The first massive polyadenylation event likely happens when the oocytes enter meiotic maturation. How polyadenylation is activated at this stage is an interesting question. In Xenopus, activation of cytoplasmic polyadenylation during oocyte maturation involves the phosphorylation of CPEB (Mendez et al., 2000a; Mendez et al., 2000b). It is still not clear what signal activates cytoplasmic polyadenylation during Drosophila oocyte maturation. Phosphorylation might also be involved in this activation. PAN GU kinase was found to activate both polyadenylation and translation of *cyclin A* mRNA during oocyte maturation (Vardy and Orr-Weaver, 2007). But it remains unclear how PAN GU activates the polyadenylation machinery and whether it activates the translation of other mRNAs in addition to *cyclin A* mRNA. It will be helpful to test if any of the proteins in the cytoplasmic polyadenylation machinery is the phosphorylation substrate of PAN GU in oocytes. Identifying other target mRNAs of PAN GU could also help to unravel the role of PAN GU in translational activation.

The second polyadenylation event is triggered by the process of egg activation. WISP is required for this polyadenylation as well. Transcripts important for early embryo development are robustly polyadenylated and translated at this time. Calcium signaling pathways are major transducers of the egg activation trigger in many systems (Ducibella et al., 2006; Horner and Wolfner, 2008; Parrington et al., 2007). Studies of the *sarah* gene (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006), which encodes the Drosophila calcipressin, indicate that proper regulation of the calcium-dependent phosphatase calcineurin by SARAH is required for egg activation in Drosopihla. SARAH function is required for lengthening of the *bicoid* mRNA poly(A) tail in early embryos (Horner et al., 2006). Other pathways also contribute to the poly(A) tail elongation of at least some mRNAs upon egg activation. Lengthening of *bicoid* mRNA poly(A) tail is affected in embryos carrying mutations in the *cortex* gene which encodes an activator of the APC/C that is required for the completion of female meiosis (Lieberfarb et al., 1996) PAN GU kinase complex regulates the translation of *cyclin B* from egg activation partially through affecting its poly(A) tail (Vardy and Orr-Weaver, 2007). It will be interesting to test if these pathways are functioning in parallel to activate polyadenylation of different sets of mRNAs or they function in the same pathway to regulate the common targets.

Target specificity in cytoplasmic polyadenylation

Because cytoplasmic polyadenylation is found to work on a variety of transcripts at different times, one question arises as to how these different targets can be discriminated in cytoplasmic polyadenylation.

One possibility could be to use different catalytic units to polyadenylate distinct RNAs because animals usually have multiple PAPs (Kwak et al., 2004). Different organisms do seem to use different combinations of these PAPs.

Differentially using different polymerases is shown in *C. elegans.* Two cytoplasmic PAPs, GLD-2 and GLD-4 are used in parallel to promote female meiotic progression (Schmid et al., 2009). The functions of these two complexes are partially redundant because their roles in meiosis converge on one common target mRNA, *gld-1*, but they clearly have different regulatory repertoires because lacking either one affects germline development. In Drosophila, my studies have demonstrated that the expression pattern of the two GLD-2 homologs is sex-specific. It is very likely that they have distinct targets; using two kinds of GLD-2-dependent polyadenylation machinery could function more efficiently to regulate the two sets of genes. It is not clear yet what PAPs are used for cytoplasmic polyadenylation in mammals. Multiple PAPs exist in mammals and they might function redundantly, which could suggest a more complicated use of PAPs in cytoplasmic polyadenylation (Nakanishi et al., 2006; Nakanishi et al., 2007).

One structural feature that distinguishes the GLD-2 proteins from regular PAPs is that GLD-2s don't have any RNA binding moiety. Because they can't bind to RNA directly, their interaction with the substrate RNA must be mediated by a separate protein or proteins. This provides a new regulatory layer to achieve differential translation of transcripts. For example, GLD-2 could be recruited to distinct groups of RNA targets by partner proteins with different RNA binding specificities. Thus by supplying or limiting specific GLD-2 partners at different times during development or in different locations in the cell, polyadenylation and activation of specific mRNAs can be regulated both spatially and temporally. A recent study has shown that switching between two GLD-2 binding partners controls the direction of gamete production in *C. elegans* (Kim et al., 2009). These hermaphrodite worms produce both types of gametes in one life cycle. The oocyte/sperm decision is made by associating GLD-2 with two different RNA binding proteins. The GLD-3/GLD-2 complex

promotes the oocyte fate while the RNP-8/GLD-2 complex promotes the sperm fate. Although it is not clear what target mRNAs are regulated by these two complexes, one reasonable hypothesis is that the two RNA binding partners differentially bind and activate oocyte-specifying mRNAs and sperm-specifying mRNAs, respectively.

In Chapter 2, I showed that WISP can interact with Bic-C, the Drosophila GLD-3 homolog, in a yeast-two hybrid experiment. Bic-C seems to have different effects on the poly(A) tail at different stages (Chicoine et al., 2007). It shortens mRNA poly(A) tails in early stages of oocytes by recruiting the CCR4 deadenylase. But later on, Bic-C's function switches to stimulating poly(A) elongation in late oogenesis. Because WISP-dependent polyadenylation happens in late oogenesis, the timing is consistent with Bic-C's role of positive regulation of poly(A) tail. It is likely that Bic-C works together with WISP to promote poly(A) tail elongation in late oogenesis.

ORB is another RNA binding protein known to interact with WISP (Benoit et al., 2008). Previous studies have shown that ORB is involved in the activation of polyadenylation and translation of two mRNAs during oogenesis, *oskar* and *cyclin B* (Benoit et al., 2005; Castagnetti and Ephrussi, 2003). My data suggests that these two mRNAs are also targeted by WISP, thus it is possible that ORB and WISP function together to elongate the poly(A) tail on target mRNAs in oocytes. Because WISP can interact with both Bic-C and ORB, the next question is whether these three proteins are present in the same complex or they form different complexes.

I have shown that WISP is involved in polyadenylation of embryonic mRNAs and it can form mRNP complexes with different mRNAs. Because WISP is not an RNA binding protein, formation of these mRNP complexes must be mediated by at least one other protein. The identities of other components in the WISP-containing mRNPs are not known yet. Analyzing what proteins are present in these mRNPs will help to further reveal the mechanism of polyadenylation regulation in Drosophila.

Xenopus studies have shown that cytoplasmic polyadenylation is mediated by CPE elements in the mRNA 3'UTR (Mendez and Richter, 2001). I performed a computational analysis to examine if WISP-regulated mRNAs are enriched for CPE elements in their 3'UTR sequences. I did not find any significant enrichment in the usage of CPE motifs in any groups of the WISP regulated mRNAs. About 25% of the maternal transcripts in Drosophila are predicted to be CPE-regulated, which is lower than the 31-40% predicted in the vertebrate genomes (Pique et al., 2008). One possibility is that Drosophila CPEs could be divergent from the ones characterized in Xenopus. Variations are often found in CPE sequences (Fox et al., 1992; Fox et al., 1989; McGrew et al., 1989) and the exact sequence of Drosophila CPE is not known yet. Using the optimized sequences of Xenopus CPEs to search Drosophila 3'UTRs possibly doesn't reflect the true usage of CPEs in Drosophila. Another possibility also exists that the WISP-dependent polyadenylation is not mediated by CPE. A recent study suggested a novel mechanism mediates cytoplasmic polyadenylation of *Toll* mRNA in Drosophila embryos (Coll et al., 2010). This polyadenylation of *Toll* in early embryos does not require the Hex and CPE elements but uses a novel region in the 3'UTR of *Toll* mRNA. My RNA immunoprecipitation experiment in Chapter 4 suggests that *Toll* mRNA is enriched in the Wisp containing RNPs in embryos of the same stage. It is possible that the novel polyadenylation sequence found in the *Toll* 3'UTR might mediate the polyadenylation by Wisp. It will be interesting to test if this novel sequence plays a role in the Wisp-dependent polyadenylation machinery.

Given that so many maternal transcripts undergo cytoplasmic poly(A) tail elongation, the possibility arises that activation of cytoplasmic polyadenylation is not only dependent on the mRNA sequences but also on their subcellular localization. In somatic cells, processing bodies (P bodies) are identified as RNA granules in the cytoplasm that are involved in the translational repression of mRNAs (Sheth and

Parker, 2003). New evidence suggests that P bodies are also present in Drosophila oocytes (Lin et al., 2008). In mouse oocytes, mRNAs are also stored in subcelluar regions such as the subcortical maternal cortex (SCMC) (Li et al., 2008). Although the function and components of these granules in oocytes are not yet clear, the possibility exists that release or recruitment of mRNAs by these granules can determine when and what maternal transcripts can by polyadenylated.

Another possibility is that the polyadenylation machinery is constitutively active in the oocytes, but a negative regulatory mechanism such as deadenylation works more on specific target mRNAs. Release of this repression would result in the activation of polyadenylation. Thus target specificity could be determined by the specific deadenylation of the target mRNA. For example, maternally supplied *hunchback* mRNA undergoes poly(A) tail elongation in the early embryo (Wreden et al., 1997). However, in the posterior of the embryo, polyadenylation is repressed by CCR4 which is recruited to the mRNA by the PUMILIO-NANOS complex (Kadyrova et al., 2007). This recruitment is mediated by the NANOS response element in the *hunchback* mRNA 3'UTR.

Polyadenylation and RNA stability in Drosophila oocytes and embryos

Length of the poly(A) tail is generally considered to be linked to the stability of the mRNA (Garneau et al., 2007). In somatic cells, a long poly(A) tail recruits the poly(A)-binding protein (PABP) which can protect the transcript from exonucleases whereas shortening of the poly(A) tail usually indicates the beginning of mRNA decay.

However, RNAs with short poly(A) tails are quite stable in Drosophila oocytes before fertilization. It is not clear how these mRNAs with short poly(A) tails are kept from degradation during early oogenesis. One possibility is that these mRNAs are protected by PABP2, a special form of poly(A) tail binding protein in the oocytes

(Benoit et al., 2005). PABP2 functions to limit the poly(A) length through deadenylation, but it might also have dual roles of protecting the mRNA from degradation by coating the poly(A) tail like the regular PABP does.

One interesting finding in my microarray study in Chapter 4 is that some maternal mRNAs are stabilized in mature oocytes in the absence of WISP function. This result suggests that RNA degradation works on at least some maternal transcripts in oocytes and this degradation requires WISP function. One explanation is that one or several components of the degradation machinery are regulated by cytoplasmic polyadenylation and they are not activated in *wisp* deficient oocytes. Another possibility is that the lack of translation of these maternal transcripts could stabilize them in the *wisp* oocytes. Maternal transcripts are actively translated in early oocytes (Matthews et al., 1989) and it has been demonstrated that active translation in cells could lead to RNA degradation (Grosset et al., 2000). In *wisp* deficient oocytes, these maternal transcripts might be stabilized because they have short poly(A) tails and are not translated.

Another interesting observation in the microarray experiment is that the accumulation of some maternal mRNAs in *wisp* mature oocytes was not detected later on in early *wisp* embryos. This suggests that egg activation triggers the degradation of the maternal transcripts and this machinery seems not dependent on cytoplasmic polyadenylation. This could be due to the activation of the RNAi pathway. It is suggested that the RNAi pathway is not active in Drosophila mature oocytes but is activated during egg activation, which leads to the degradation of some maternal transcripts (Kennerdell et al., 2002).

Although egg activation seems to trigger the degradation of some maternal mRNAs in *wisp* deficient eggs, many maternal mRNAs remain stable in these embryos for hours after fertilization (Tadros et al., 2003). This suggests that the major

RNA degradation machinery that clears maternal transcripts in early embryos is probably not activated in *wisp* deficient embryos. In Drosophila, shortly after egg activation, the CCR4 deadenylase is recruited to the maternal mRNAs by a RNA binding protein SMAUG, which leads to shortening of the poly(A) tail and degradation of the transcript (Bashirullah et al., 1999; Semotok et al., 2005). Deadenylation-coupled degradation is a major mechanism that destabilizes about 1600 maternal mRNAs stored in the developing embryo so that the transcription of the zygotic genome can be activated (Tadros et al., 2007). Although *smaug* mRNA is a target of cytoplasmic polyadenylation (my microarray result), polyadenylation of its mRNA is not required for translation of *smg* mRNA in the activated eggs (Tadros et al., 2007). In Chapter 3, I have also shown that SMAUG protein level is normal in the *wisp* deficient embryos. Thus lack of SMAUG protein is less likely the reason of stabilization of maternal transcripts in *wisp* deficient embryos. It is possible that one or several other components in the degradation machinery are not active in the absence of WISP function. Activation of these components could be regulated by WISP.

Another possibility is that the elongation of mRNA poly(A) tail during late oogenesis or egg activation can itself be required for RNA degradation later in the embryo. The elongation of the poly(A) tail can probably exclude some oocyte factors such as PABP2 from the transcripts thus setting up the stage for the degradation later on in the embryo. The SMAUG protein can then recruit CCR4 to shorten the poly(A) tail. This time the short tails are not protected and lead to degradation.

Polyadenylation and protein translation

In general, mRNAs with a short poly(A) tail are dormant in translation, while elongation of polyadenylation correlates with activation of translation. It has been shown that a long poly(A) tail can help to enhance translation efficiency by interacting

with the 5' cap of the mRNA and stabilizing translational initiation factors (Preiss and Hentze, 1998; Tarun and Sachs, 1995).

However, the correlation between poly(A) tail length and translation is not always perfect. In Drosophila oogenesis, it has been observed that polyadenylation of several maternal transcripts is not absolutely required for their translation. For example, translation of *smaug* does not need a long tail, although *smaug* mRNA is regulated by cytoplasmic polyadenylation (Tadros et al., 2007). Polyadenylation is not required for the translation of *cyclin B* (Vardy and Orr-Weaver, 2007). *cyclin B* mRNAs have short poly(A) tails in *orb* mutant embryos, but Cyclin B protein levels are normal. While in many other cases, cytoplasmic polyadenylation is required for translation. For example, *bicoid, oskar* and *nanos* mRNAs are not translated in *wisp* deficient embryos (Benoit et al., 2008). Thus what role cytoplasmic polyadenylation plays in translation remains an open question.

In many cases, translational regulation is determined by multiple regulatory mechanisms that function together on the same transcript. Translational repression relies on both deadenylation of the mRNA and the binding of translational repressors. Translation activation happens upon both poly(A) tail elongation and the release of translational repressors.

In Drosophila embryos, translational regulation of several axis patterning genes are through both poly(A)-dependent and poly(A)-independent mechanisms. For example, *nanos* mRNA is uniformly distributed in the bulk cytoplasm but its translation is repressed (Gavis and Lehmann, 1994). This translation repression is mediated by SMAUG, which recognizes the SMAUG response element (SRE) in *nanos* 3'UTR and recruits the CCR4/POP2/NOT deadenylation complex to shorten the poly(A) tail of the mRNA (Dahanukar et al., 1999; Smibert et al., 1996). Meanwhile SMAUG also recruits CUP which interferes with the translation initiation

factor eIF4E (Nelson et al., 2004). In the posterior pole, localized OSKAR protein prevents the binding of SMAUG and the CCR4 deadenylation complex, which in turn allows the translation of *nanos* mRNA (Zaessinger et al., 2006).

Posttranscriptional regulation of maternal mRNAs is a complicated system. In many cases, several mechanisms function together on the same mRNA to control its translation. Using multiple regulatory mechanisms could be the strategy that animals use to ensure the precise expression of genes with important developmental functions. This complexity in regulation is crucial for maternal mRNAs because these mRNAs need to be activated at different times and locations during development. Cytoplasmic polyadenylation is an important part in the tool kit of posttranscriptional regulation. The discovery of WISP, a major component of cytoplasmic polyadenylation machinery, will help to tease apart the multiple layers in posttranscriptional regulation of maternal mRNAs. Identifying genes whose mRNAs are regulated by cytoplasmic polyadenylation during oogenesis or upon egg activation will also help to understand whether and how products of these genes play roles in oogenesis and egg activation. Future studies on the functions of these genes could help to further understand oogenesis and embryo development in Drosophila.

APPENDIX A

MAPPING LAMIN INTERACTION MOTIFS IN YOUNG ARREST PROTEIN

A.1 Introduction

Initiation of embryogenesis is under the control of developmental cues from both parents. Several mutants whose effects are detected after sperm entry into the egg have been isolated. For example, *ms(3)sneaky (snky)* and *misfire (mfr)* mutations both affect sperm head decondensation in the egg by presumably preventing removal of the sperm plasma membrane in the fertilized egg (Fitch and Wakimoto, 1998; Ohsako et al., 2003). The paternal effect mutation *ms(3)K81(K81)* prevents the incorporation of the paternal chromosomes into the zygote nucleus, while the maternal effect mutation *maternal haploid (mh)* results in a similar phenotype (Loppin et al., 2001; Yasuda et al., 1995). Several Drosophila maternal effect genes are also important in the first zygotic division. *sesame (ssm)* is a maternal-effect mutation in the histone chaperone HIRA which affects the formation of male pronuclei (Loppin et al., 2005a). KLP3A coding for a kinesin-like protein affects the migration of the female pronucleus (Williams et al., 1997). Three genes *giant nuclei (gnu), pan gu (png),* and *plutonium (plu)* are needed for the gap-phase free cell cycles in early embryos. They function as a kinase complex and their respective maternal effect mutations cause the uncoupling of S and M phases and produce giant nuclei in early embryos (Lee et al., 2003). Finally, mutations in *fs(1) Young arrest (Ya)* gene form pronuclei but do not enter the first mitotic division (Lin and Wolfner, 1991; Sackton et al., 2009).

YA protein is only detected in ovaries and early embryos. Homozygous mutant flies are perfectly normal except that the females are sterile (Lin and Wolfner, 1991). *Ya* is a strict maternal effect gene because a wild type copy from the paternal genome

cannot rescue the mutant phenotype in embryos from homozygous mutant females. Female meiosis completes normally in embryos from homozygous Ya^2 mutant females (referred to as Ya^2 embryos hereafter) but arrest occurs before the gonomeric mitosis (Sackton et al., 2009). Mutant embryos have abnormally condensed chromatin, and different haploid female meiotic products can have different condensation states.

YA is located in nuclear envelopes of zygotes during interphase (Yu et al., 1999). The nuclear lamina is a protein network underlying the inner nuclear membrane. Lamin, an intermediate filament protein, is the major constituent of the nuclear lamina. Drosophila has two lamin genes, *lamin* Dm_0 and *lamin C*. *Lamin* Dm_0 is expressed at all developmental stages and the protein is found in all nuclei except mature oocytes and sperm. Yeast two-hybrid assays showed that YA interacts with Lamin Dm_0 (which I will refer to as "Lamin" hereafter) through YA's carboxyl terminal region (Goldberg et al., 1998). YA's C-terminal region is sufficient to target YA protein to the nuclear envelope (Mani et al., 2003). It was also found that this C-terminal region is one of the four domains of YA that bind to DNA and Histone H2B (Yu and Wolfner, 2002). Previous studies of a deletion of this C-terminal region showed that it is important for YA's function; however, deleting the entire C-terminal region doesn't address whether the loss-of-function is due to loss of lamin interaction or a defect in DNA binding or some other functions. Here I report a yeast two-hybrid study that maps the essential amino acids in YA's lamin-interaction domains.

A.2 Materials and Methods

Yeast two-hybrid assay: Fragments of YA C-terminus and full-length Lamin Dm_0 were generated by PCR and then cloned into vectors of the ProQuest yeast two-hybrid system (Invitrogen, Carlsbad, CA). Primers used are listed in Table A.1. In–frame fusions of each coding region were generated in both the DNA-binding domain

Table A.1 Sequences of PCR primers.

Primer	Sequence 5' -> 3'
YA-1	CACCATGTCGTTTTCCAATGTCCTAATC
YA-2	CTACTGGCGACGCATGCGC
YA-3	CACCCTGAGCGGCGAGTTTTCGC
YA-4	CTACCTTGCACTGGGGCGC
YA-5	CACCGTGGGCGAGCCGGAG
YA-6	CTACAAGCTGGCCGGCAGTC
YA-7	CACCCGCGGCGAACGCG
YA-8	CTAATCCTGCACGCCGCTATAGA
YA-9	CACCGACTCCTCCATGGAGGACCAG
YA-10	CTAGGGCGGCGTCTCGAA
YA-12	CTACTGGTCCTCCATGGAGGAG
YA-21	CTACCTGGGCCGGATGCG
YA-22	CTAGCTGGACGGCGACAGC
YA-91	CACCAAGAGAAAGCGCACTCAGACGC
YA-92	CACCCAGTACTGTAGCCCGCTGTCG
Lamin-1	CACCATGTCGAGCAAATCCCGACG
Lamin-2	TTACATAATGGCGCACTTCTCG

vector pDEST32 and the activation domain vector pDEST22. Haploid yeast cells transformed with pDEST22 derivatives were mated with yeast cells of the opposite mating type transformed with pDEST32 derivatives. Diploid cells were grown on -Trp -Leu synthetic medium and tested for growth on -Trp -Leu -His synthetic media containing 3-Amino-1,2,4-triazole (3-AT).

DNA mutagenesis: Random DNA mutagenesis by GeneMorph II random mutagenesis kit (Stratagene, La Jolla, CA) was used to generate a mutagenized DNA library of YA fragment corresponding to residues 506-696. Quickchange II site-directed mutagenesis kit (Stratagene) was used to generate point mutation in the YA fragment (residues 506-696). Primer sequences were as followings:

Random upper 5' GTGGTCGACCGCCCCCTTCACC

Random lower 5' GTGGAATTCACCCGCGCGGCTA

P689L 5' CTACTGGCGACGCATGCGCTCGATGAGCTCCCTGGGCC

P689H 5' CTACTGGCGACGCATGCGCTCGATGTGCTCCCTGGGCC

Reverse yeast two-hybrid screen: A randomly mutagenized DNA library of YA fragment corresponding to residues 506-696 was co-transformed into MaV203 cells (Invitrogen) with linearized pDEST32 vectors carrying the wild-type YA fragment. This co-transformation generated a library of pDEST32 derivatives containing potentially mutant YA sequences by gap repair in the yeast cells (Vidal et al., 1996a). The pDEST22 vector containing an in-frame fusion of Lamin Dm_0 was then transformed into these yeast cells. Yeast cells were grown on -Trp -Leu synthetic medium and then transferred to -Trp -Leu selective media containing 5-Fluroorotic acid (5FOA) to test for YA-Lamin interaction. Cells that survived on the 5FOA selective media were cultured. BD-vectors were then purified from these cells. Insertions in these vectors were sequenced to determine the mutation in the YA fragment by sequencing.

A.3 Results

YA interacts with Lamin through two separate domains in its C-terminus: Previous yeast two-hybrid study showed that YA binds to Lamin through its C-terminal 191 amino acids (residues 506-696) (Goldberg et al., 1998) and the last 90 amino acids (residues 607-696) in this region are sufficient to target GFP-NLS to the nuclear envelope in S2 cells (Mani et al., 2003). To map further the essential motifs in the Lamin-interaction domain, I created a series of short fragments in the last 90 amino acids of the YA C-terminus and tested if they can interact with Lamin using yeast two-hybrid assay. Two fragments corresponding to residues 648-696 and 658-696, respectively, can interact with Lamin while another two smaller regions corresponding to residues 668-696 and 658-686, respectively, do not interact with Lamin (Figure A.1). These results suggest that the region corresponding to residues 658-696 is the essential domain for YA-Lamin interaction. By testing the other regions in the C-terminal 191 amino acids, I also found that the region corresponding to residues 506-606 is able to bind Lamin in yeast two-hybrid assay while two smaller fragments 506-555 and 556-606 lost Lamin-binding ability (Figure A.1). This suggests that the first 101 amino acids of YA's C-terminus forms another Lamin-binding domain. In summary, I found that YA interacts Lamin through two separate domains (domain 1: residues 506-606; domain 2: residues 658-696) in its C-terminus region. Either one of these two domains is sufficient for binding of YA to Lamin in yeast two-hybrid.

Pro^{689} is a critical amino acid in the second Lamin binding domain: To further analyze YA-Lamin interaction, I used an interaction trap approach to identify amino acids in the YA's C-terminus region that are critical for the interaction. Reverse yeast two-hybrid is a modification of the traditional yeast two-hybrid system to detect dissociation of protein-protein interaction (Vidal et al., 1996a; Vidal et al., 1996b).

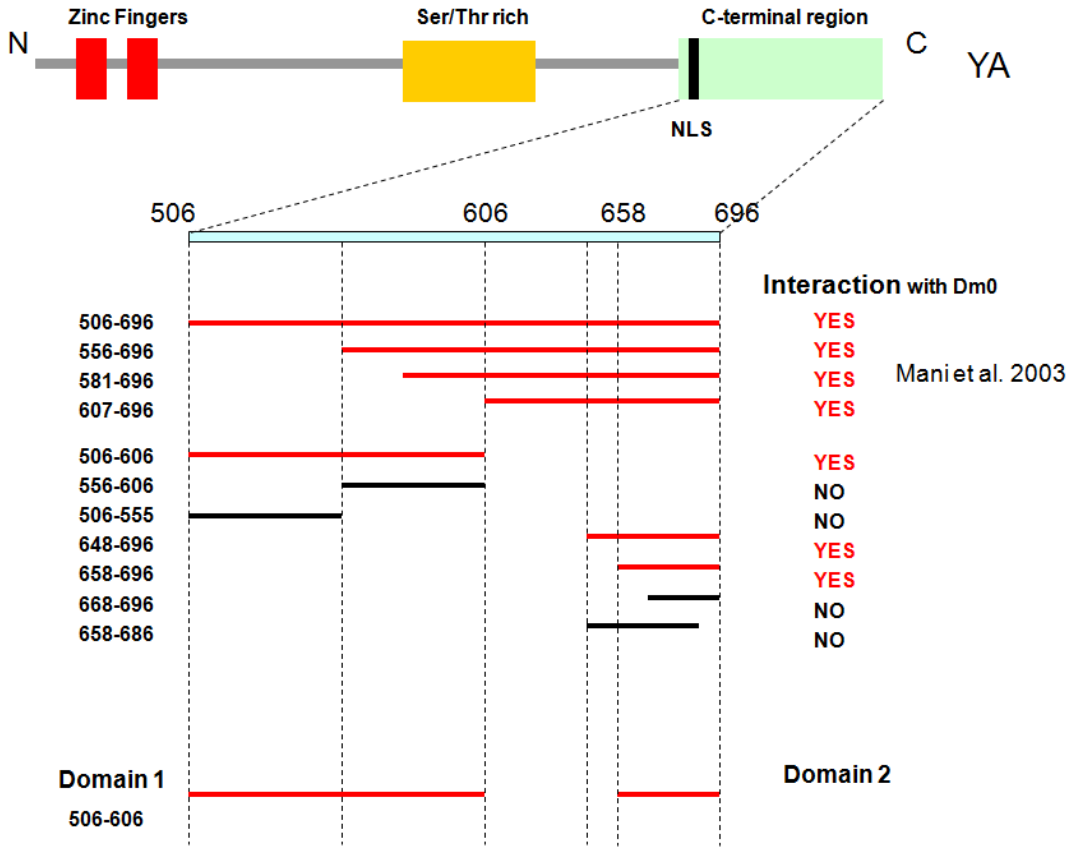


Figure A.1 YA's Lamin-interaction domains. Deletion analysis was used to map Lamin-interaction domains in the YA C-terminus (residues 506-696). Fragments that interact with Lamin are shown in red while fragments that do not interact are shown in black.

The wild-type form of AD-Lamin fusion protein was tested for interaction with a library of potential mutants of the YA protein. If the two test proteins can still interact with each other, the report gene, in this case *URA3,* will be turned on to kill the host cell in the presence of a drug 5FOA. However, if a mutation in the YA protein disrupts the interaction, the host cell will survive (Figure A.2A). Mutations were generated in the YA's C-terminus (residues 506-696) by random mutagenesis PCR and mutants that prevent binding to Lamin were identified by selecting for URA^- colonies on the selective medium containing 5FOA.

Out of the ~10,000 transformants tested, 41 colonies were found to be 5FOA-resistant. Mutant YA DNAs were recovered from these colonies and 26 unique mutations were identified. Among these 26 mutants, 12 carry frame-shift mutations and another 12 carry nonsense mutations. Both of these two groups of mutations resulted in truncated YA proteins (ends indicated by vertical lines in Figure A.2B). Interestingly, none of these truncated forms is longer than the first 101 amino acids of the YA C-terminus, which is consistent with the result from the deletion analysis that these 101 amino acids are sufficient in YA-Lamin interaction.

The last two YA mutants carry point mutations that change the same Proline at residue 689 to Leucine or Histidine, respectively. This amino acid Pro^{689} is in the second Lamin-interaction domain of YA protein that was identified by the deletion analysis. This proline is conserved in YA orthologues of all 12 Drosophila species whose genomes have been sequenced, which suggests a conserved function of this amino acid. Substitution of this amino acid could cause protein structure change to disrupt YA's function, presumably its interaction with Lamin. To test how the substitution of Pro^{689} affects YA-Lamin interaction, the two point mutations were introduced by site-directed mutagenesis into whole YA-C segment (residues 506-696) or a smaller segment just containing the Lamin-interaction domain 2 (residues 658-

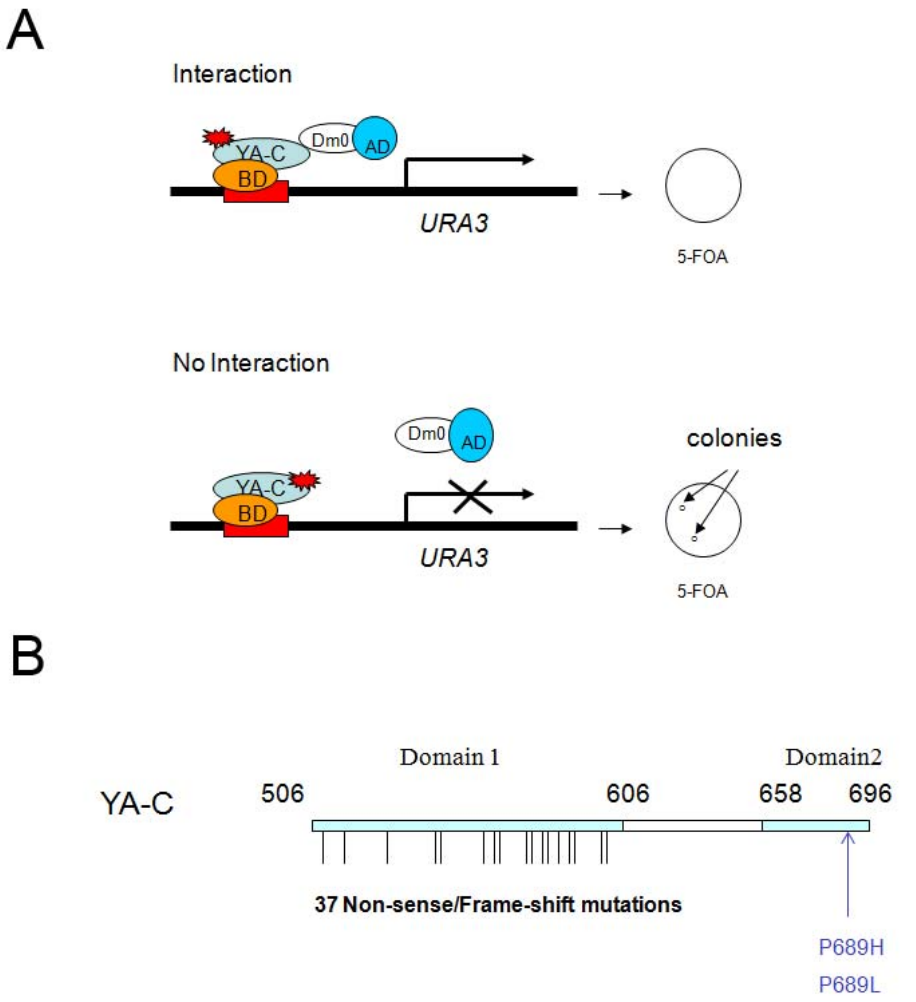


Figure A.2 Mutations in the YA C-terminus that disrupt Lamin interaction. (A) A strategy to screen for mutations in the YA protein that abolish interaction with Lamin using yeast two-hybrid assay. (B) Summary of mutations in the YA C-terminus that abolish the interaction with Lamin. End points of truncated proteins caused by non-sense and frame-shift mutations are indicated by vertical bars and single amino acid substitutions are shown by arrow.

696) and re-tested for interaction with Lamin using the standard two-hybrid method. Both mutations prevented the segment containing only domain 2 from interacting with Lamin, but did not affect the whole YA-C segment probably because it can still interact with Lamin through its intact interaction domain 1 (Table A.2). This suggests that Pro^{689} is critical for Lamin binding of domain 2.

Table A.2 Interaction between Lamin Dm_0 and YA Pro^{689} mutants in yeast two-hybrid assay.

WT: wild type

AD: activation domain

BD: DNA binding domain

+: growth on -Trp -Leu -His media supplied with 3-AT

-: no growth on -Trp -Leu -His media supplied with 3-AT

YA fragment in BD fusion	AD-Lamin Dm_0
506-696 (WT)	+
506-696 (P689H)	+
506-696 (P689L)	+
658-696 (WT)	+
658-696 (P689H)	-
658-696 (P689L)	-

APPENDIX B

YOUNG ARREST PROTEIN INTERACTS WITH DROSOPHILA POLO KINASE

B.1 Introduction

Polo-like kinases are highly conserved in eukaryotes and have multiple functions in both mitosis and meiosis. Polo-like kinases play crucial roles in different stages of cell cycle progression, such as mitosis entry, spindle formation, chromosome segregation, cytokinesis and mitosis exit (Barr et al., 2004). Xenopus Polo-like kinase (Plx-1) plays a major role in egg activation by triggering exit from meiosis II when the calcium increase is induced by fertilization (Rauh et al., 2005). In Drosophila, mutations in the *polo* gene result in multiple defects in female meiosis and arrest in early embryogenesis (Clarke et al., 2005; Llamazares et al., 1991; Riparbelli et al., 2000). POLO kinase activity is needed during meiosis to regulate the localization of a centromere cohesion protein MEI-S332 for proper chromosome segregation (Clarke et al., 2005).

B.2 Materials and Methods

Yeast two-hybrid assay: Fragments of YA, Drosophila POLO and Xenopus Plx1 were generated by PCR and then cloned into vectors of the ProQuest yeast two-hybrid system (Invitrogen, Carlsbad, CA). In–frame fusions of YA fragments were generated in the DNA-binding domain vector pDEST32. POLO and Plx1 were generated as in-frame fusions in the activation domain vector pDEST22. Haploid yeast cells transformed with pDEST22 derivatives were mated with yeast cells of the opposite mating type transformed with pDEST32 derivatives. Diploid cells were

grown on -Trp -Leu synthetic medium and tested for growth on -Trp -Leu -His synthetic media containing 3-Amino-1,2,4-triazole (3-AT).

Site-directed DNA mutagenesis: Quickchange II site-directed mutagenesis kit (Stratagene) was used to generate point mutation in the YA gene. Sequences of mutagenesis primers were as followings:

S226F 5' CATACGCTCGACGTTCACACCACTGCACG

T227A 5' CGCTCGACGTCCGCACCACTGCACG

S338A 5' CGATCTCAGCGAGCACGCTTCGCACGAGGCAGCA

S364A 5' ACCCAGAGCGAGTCCGCCGCCTCGGAGGTCAA

S650A 5' AGTGCAAGGGACTCCGCCATGGAGGACCAGC

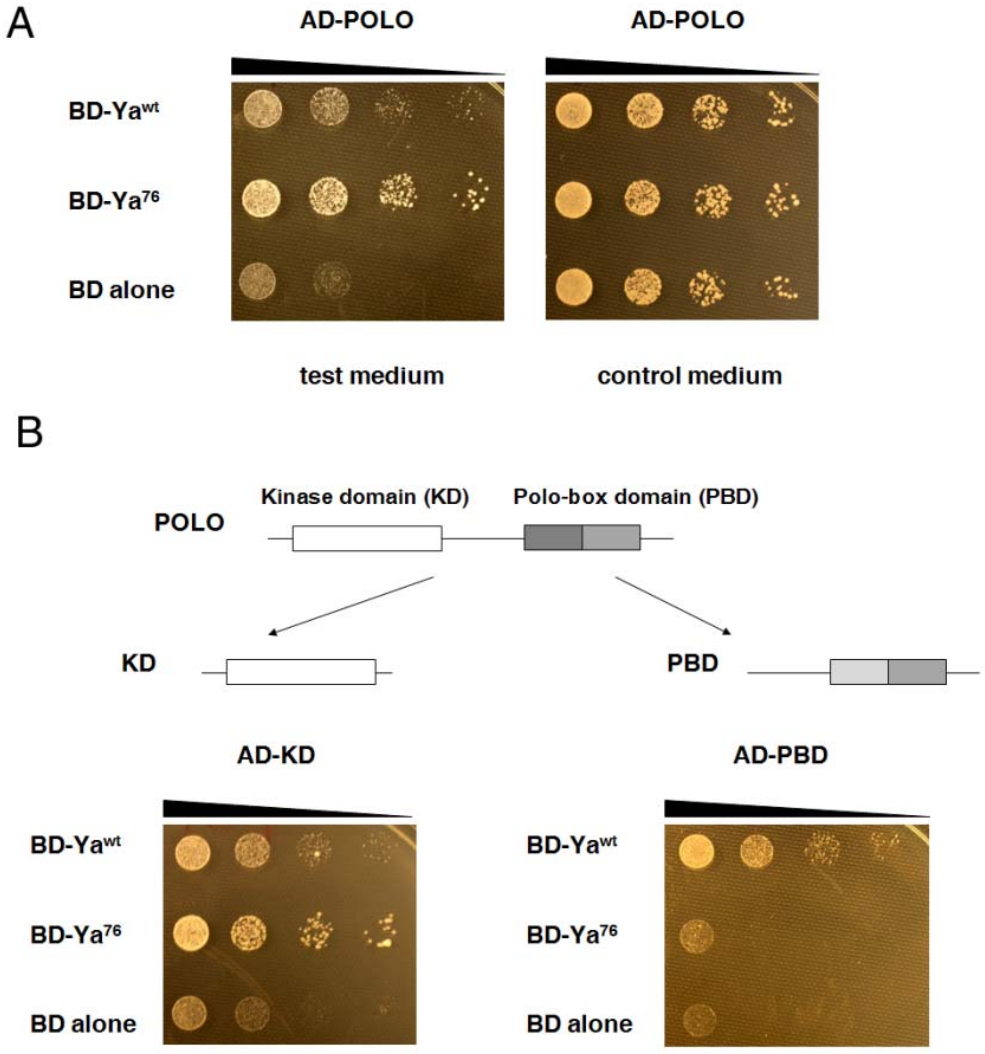
B.3 Results

YA can interact with POLO kinase in yeast two-hybrid assay: Using yeast two-hybrid assays, I found that YA can interact with POLO (Figure B.1A). Full-length POLO in an activation domain (AD) fusion can interact with wild-type YA in a binding domain (BD) fusion but not with the BD alone (that is, an empty vector).

Polo-like kinases consist of a catalytic kinase domain (KD) and a noncatalytic C-terminal region, called Polo-binding domain (PBD) which recruits the kinase to the target proteins by recognizing a phosphoserine/phosphothreonine containing motif in the target protein (Elia et al., 2003). Yeast two-hybrid assay showed that each of the two main domains of POLO is sufficient to interact with YA (Figure B.1B). This result suggests that YA can be one of POLO's targets.

Substitution of Serine^{226} abolishes YA's ability to interact with POLO PBD: Four *Ya* mutant alleles were previously isolated from EMS mutagenesis screens, including two strong, apparent null, alleles, Ya^{2} and Ya^{76} and two leaky alleles, Ya^{70} and Ya^{77}(Liu et al., 1995). One of the alleles, Ya^{76}, is a Serine-to-Phenylalanine

Figure B.1 YA interacts with POLO. (A) YA and YA^{76} are tested in yeast two-hybrid assay for interaction with POLO. Yeast cells containing both AD and BD fusion proteins were spread in 1 to 10 dilution series from left to right on non-selective medium or medium supplied with 3-AT. (B) Yeast two-hybrid assay was used to test the interaction between different forms of YA and POLO's two major functional domains. PBD, Polo-binding domain; KD, kinase domain.


A
AD-POLO
AD-POLO
BD-Ya^wt
BD-Ya^76
BD alone
test medium
control medium
B
POLO
Kinase domain (KD)
Polo-box domain (PBD)
KD
PBD
AD-KD
AD-PBD
BD-Ya^wt
BD-Ya^76
BD alone
BD-Ya^wt
BD-Ya^76
BD alone

substitution at residue 226. Previous studies by Liu et al., 1995 have shown that most *Ya*^{76} mutant embryos are arrested at the pronuclear stage while YA^{76} protein from these mutant embryos has normal size and abundance as wild type, which suggests that this amino acid substitution could disrupt the function, but not the gross structure, of the YA protein.

Ser^{226} is in a region that is conserved among YA orthologues of the twelve Drosophila species whose genomes have been sequenced (Figure B.2). This suggests that this region in the YA proteins could have a conserved function. Analysis of the primary amino acid sequence of this region indicates that it contains a putative binding motif that matches the consensus binding sequence of Polo-like kinases (Elia et al., 2003) and Ser^{226} is the core residue in the motif. Using yeast two-hybrid assays, I found that the substitution of Ser^{226} in YA^{76} abolishes its binding with POLO's PBD region (Table B.1). Changing an adjacent Threonine at residue 227 to an Alanine shows the same result as the YA^{76} allele in binding to POLO. These results suggest that POLO's PBD region interacts with YA through the motif surrounding Ser^{226}.

Although YA^{76} does not interact with POLO's PBD, it can still interact with POLO's KD region (Table B.1). This interaction suggests that YA could be a phosphorylation substrate of POLO. A phosphorylation site for Polo-like kinases has been identified as the Serine or Threonine in the consensus sequence D/E-X-S/T-φ-X-D/E, in which X stands for any amino acid and φ stands for a hydrophobic amino acid (Nakajima et al., 2003). By scanning the primary amino acid sequence of YA, I found three Serines at residues 338, 364 and 650 respectively match the consensus phosphorylation site. I made a transgenic fly line containing a mutagenized form of YA carrying Alanine substitutions of all these three Serines (refer to as "All-A") and used complementation test to check if this mutant form is functional in the absence of wild-type YA protein. Ectopic expression of this All-A form failed to complement YA

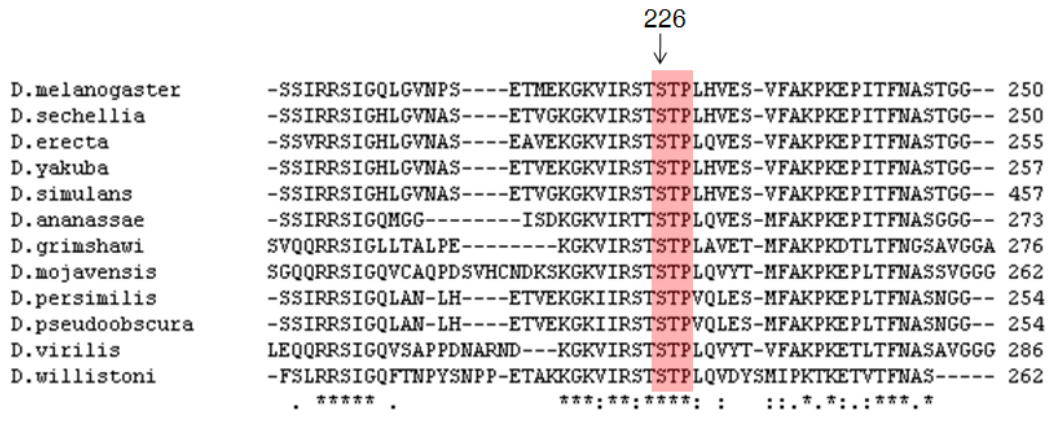


Figure B.2 Alignment of YA proteins of 12 Drosophila species. Amino acid sequences of the predicted YA proteins of 12 Drosophila species were aligned against each other and the region containing a motif that binds POLO is shown. Identical or similar residues between the sequences are indicated with an asterisk or a dot (or dots), respectively. The conserved Serine-Threonine-Proline triple amino acids which bind POLO are highlighted in the red box. Arrow indicates the amino acid change in the YA^{76} allele of D. melanogaster.

Table B.1 Interaction between YA and Polo-like kinases in yeast two-hybrid assay.

AD: activation domain; BD: DNA binding domain; WT: wild type; FL: full length; PBD: Polo-binding domain; KD: kinase domain

+ : weak interaction; ++ : intermediate ineraction; +++ : strong interaction; - : no interaction

BD fusion	AD fusion			
	Plx1	POLO	POLO(PBD only)	POLO(KD only)
Empty vector	-	-	-	-
YA (WT)	++	+	++	++
YA^{76}	++	++	-	+++
YA (T227A)	++	++	-	+++
YA (S226E)	++	+	-	+++
YA (T227E)	++	++	-	+++
YA^{2}	-	-	-	-
YA^{70}	+++	++	+	+++
YA-C (506-696)	+++	+	-	+++

mutant alleles. This result suggests that these three amino acids are important for YA function. Changing Serines to Alanines probably abolishes the phosphorylation by POLO.

In summary, POLO interacts and probably phosphorylates YA protein. This interaction happens in two consecutive steps. First POLO is recruited to YA through the interaction between the PBD of POLO and a YA region in which Ser^{226} and Thr^{227} are the core amino acids in the motif. This recruitment brings together POLO's KD and YA so that POLO can phosphorylate the substrate amino acids in the YA protein.

APPENDIX C

USING DROSOPHILA AS A MODEL SYSTEM TO STUDY HUMAN LMNA MUTATIONS[3]

C.1 Introduction

The nuclear lamina is a cross-linked proteinaceous layer that underlies the inner nuclear membrane, which has been suggested to have various functions such as the maintenance of nuclear shape and organization of nuclear pores as well as regulating transcription or DNA replication (Gruenbaum et al., 2005). Main protein components of the lamina network are lamins and lamin-binding proteins.

Lamins, which are intermediate-filament proteins, form a meshwork underneath the nuclear envelope. Based on the biochemical properties and expression patterns, lamins are divided as two types, A- and B- types (Hutchison, 2002). Unlike B-type lamins which are ubiquitously expressed in all cell types and are essential for viability, A-type lamins are expressed in certain tissues and are not required for viability. However, mutations in A-type lamins have been associated with a range of human diseases known as laminopathies, such as Emery-Dreifuss muscular dystrophy (EDMD), Dunnigans's familial partial lipodystrophy, Charcot-Marie Tooth syndrome type 2, and Hutchinson-Gilford progeria syndrome (HGPS) (Bonne et al., 2000; Chaouch et al., 2003; Eriksson et al., 2003; Shackleton et al., 2000).

In humans, *LMNB1* and *LMNB2* encode B-type lamins while *LMNA* is the only gene encoding A-type lamins. The *LMNA* gene generates two isoforms, lamin A and lamin C, by alternative splicing. Some of the laminopathies are associated with

[3] LMNA mutation constructs are made by J. Liu, and transgenic fly lines are generated by C. Perez and N. Buehner.

mutations in the *LMNA* gene, but it is still not clear how these mutations in one gene can cause various disease phenotypes.

It will be useful to have an in vivo animal system to study how *LMNA* mutants cause disease in vivo. Drosophila has been used as a model system to understand developmental processes in vertebrates because there are powerful genetic tools available in Drosophila. And Drosophila also has two types of lamins, A-type encoded by *Lamin C (LamC)* and B-type encoded by *Lamin* Dm_0, which makes it a potential model system to study human LMNA mutants. I tested if Drosophila can be used as a model system to study human laminopathies.

C.2 Materials and Methods

Ectopically expressing human *LMNA* in Drosophila: UAS-GAL4 system was used to drive ectopic expression of transgenic *LMNA* proteins in the whole flies. Transgenic fly lines carrying a wild-type human *LMNA* gene or a mutant form of *LMNA* were generated using the standard P-element insertion method. These transgenic flies were then crossed to a *tubulin-GAL4* line to drive ubiquitous expression of the transgene. Stage specific expression was driven by crossing the transgenic flies to the *hsp70-GAL4* driver line. Expression was triggered by incubating the adult flies at 37 °C for 1 hour.

Western blot analysis: Whole fly extracted were prepared from adult flies 24 hours after heat-shock treatment and were then separated on a SDS-PAGE gel. Anti-LMNA antibody (Millipore, Billerica, MA) was diluted 1:50 for probing the blot.

Immunofluorescence analysis of male accessory glands: Accessory glands were dissected from 3- to 4-day-old virgin males and fixed in 4% paraformaldehyde. Fixed glands were washed in 1 X PBST (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Triton X-100) and incubated at 4°C overnight with

a 1:50 dilution of a rabbit anti-LMNA antibody or with a 1:50 dilution of a rabbit anti-Lamin Dm_0 antibody in 1 X PBST. RNaseA (Roche Applied Science, Indianapolis, IN) was added to a final concentration of 5 μg/ml. Secondary antibody [Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:200] was then added for 2 hr at room temperature. Propidium iodide (Invitrogen) was added at a concentration of 10 μg/ml to stain DNA. Samples were mounted in 75% glycerol containing 940 mM *n*-propyl. Staining in fertilized and unfertilized eggs was analyzed using confocal microscopy [Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope (Leica Microsystems, Germany)].

C.3 Results

Ubiquitous expression of the human *LMNA* gene causes lethality: To express ectopically the human *LMNA* gene in Drosophila, wild-type and disease forms (listed in Table C.1) of *LMNA* were cloned by J. Liu, and transgenic UAS-*LMNA* fly stocks were generated by C. Perez and N. Buehner. Flies carrying the transgenic *LMNA* gene were crossed to *tubulin-GAL4/Sb* flies which can drive ubiquitous expression of the transgene. For each line, progeny from five mating pairs were collected for three days and counted. For all the crosses, 100% of the progeny were Sb (as control, *UAS-LMNA/Sb*) flies and I never observed non-Sb (*UAS-LMNA/ tubulin-GAL4*) adult flies (Figure C.1). This result suggests that ubiquitously expressing wild-type or mutant forms of the human *LMNA* gene in Drosophila causes lethality during development. This result is not surprising because the *tubulin* driver generally drives expression ubiquitously and given the tissue specific expression pattern of A-type lamins during development, it is possible that expression of *LMNA* needs to be regulated. Another possibility is that although both Drosophila *LamC* and human

Table C.1 Human LMNA mutations.

Construct	LMNA form	Associated Disease
pJKL633	wild type	normal
pJKL634	L85R	Cardiomyopathy
pJKL635	N195K	Cardiomyopathy
pJKL636	E358K	Emery Dreifuss Muscular Dystrophy
pJKL637	M371K	EDMD
PJKL638	R386K	EDMD
pJKL639	R482W	Lipodystrophy
PJKL640	L530P	EDMD
pJKL642	Progeria	Hutchinson- Gifford

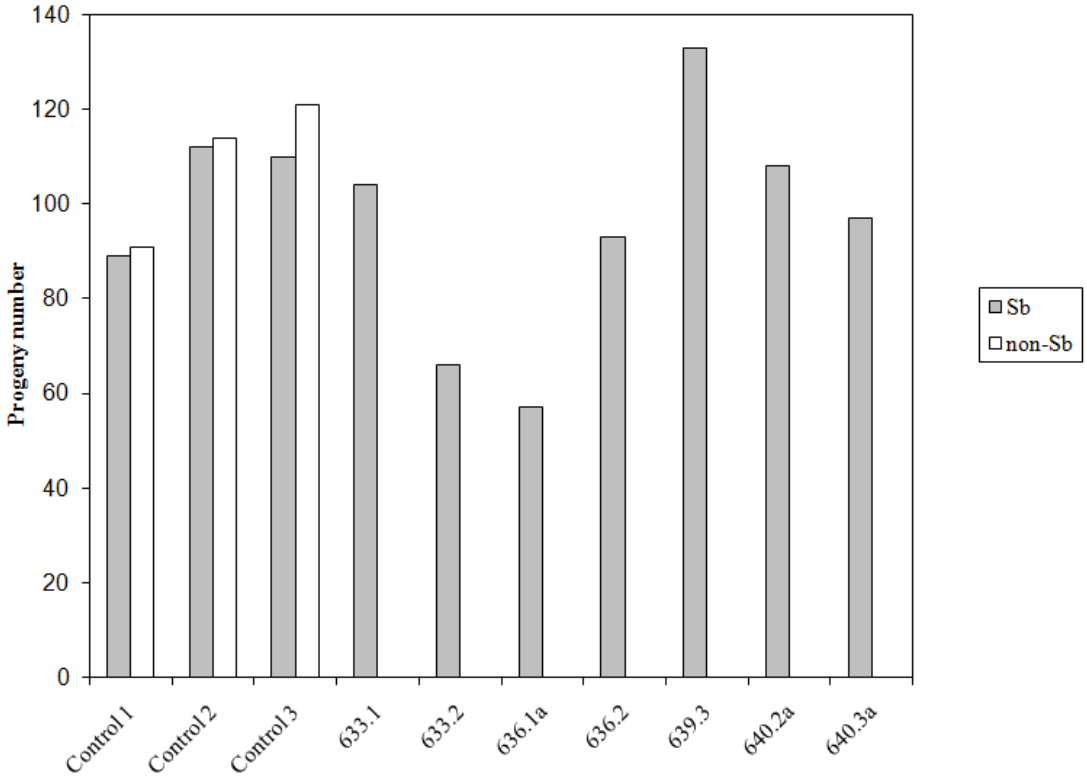


Figure C.1 Ubiquitous expression of human LMNA causes lethality. *UAS-LMNA/UAS-LMNA* flies were crossed with *tubulin-GAL4/Sb* in single mating pairs. Progeny of five mating pairs for each cross were counted over a period of 3 days. Control lines are with empty vectors.

LMNA encode A-type lamins, their primary protein sequences are quite divergent and they might have different functions.

Human *LMNA* gene can be expressed transiently in adult flies: To regulate the expression of the human *LMNA* gene in adult flies, *LMNA* transgenic fly stocks were crossed to *hsp70-GAL4/CyO* flies which drive ectopic expression of the transgene only after heat-shock treatment. For each line, both CyO (as control, *UAS-LMNA/CyO*) or non-CyO (*UAS-LMNA/hsp70-GAL4*) progeny were collected and aged for 3-4 days. Protein expression was induced by incubating the adult flies at 37°C for 1 hour and Western blot analysis was performed on whole fly protein extract 24 hours after heat-shock treatment using an anti-LMNA antibody. Analysis of the *LMNA* mutant 638 (R386K) was shown as an example in Figure C.2. Human LMNA protein was only detected in *UAS-LMNA/hsp70-GAL4* flies after heat-shock treatment (arrow), but not in *UAS-LMNA/CyO* control flies. Weak signal detected in *UAS-LMNA/hsp70-GAL4* flies before heat-shock induction is due to background leaky expression.

Human *LMNA* proteins are localized to the nuclear periphery in Drosophila cells: Male accessory glands were used to examine the nuclear localization of ectopically expressed lamin proteins because nuclei in this tissue are large, flat, and easy to visualize. Glands of *UAS-LMNA/hsp70-GAL4* male flies expressing the wild-type form of the human *LMNA* gene were dissected at 24-hr after heat-shock induction and were then stained with propidium iodide to visualize DNA and with anti-LMNA or anti-Lamin Dm_0 antibody to visualize lamin proteins.

In 19% (n=16) of the glands, *LMNA* staining was observed at the nuclear periphery (Figure C.3A). This pattern resembles the nuclear localization of ectopically expressed wild-type Drosophila LamC in salivary glands (Schulze et al., 2005). However, in the remaining 81% of the glands, I observed strong aggregation of *LMNA* at the nuclear periphery (Figure C.3B, arrows). This aggregation phenotype was also

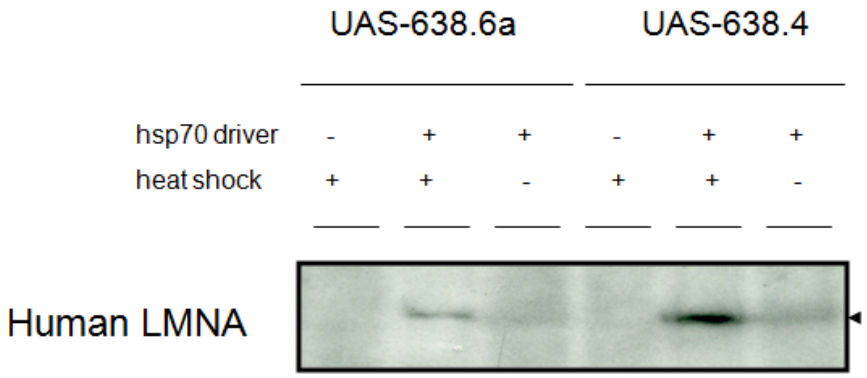
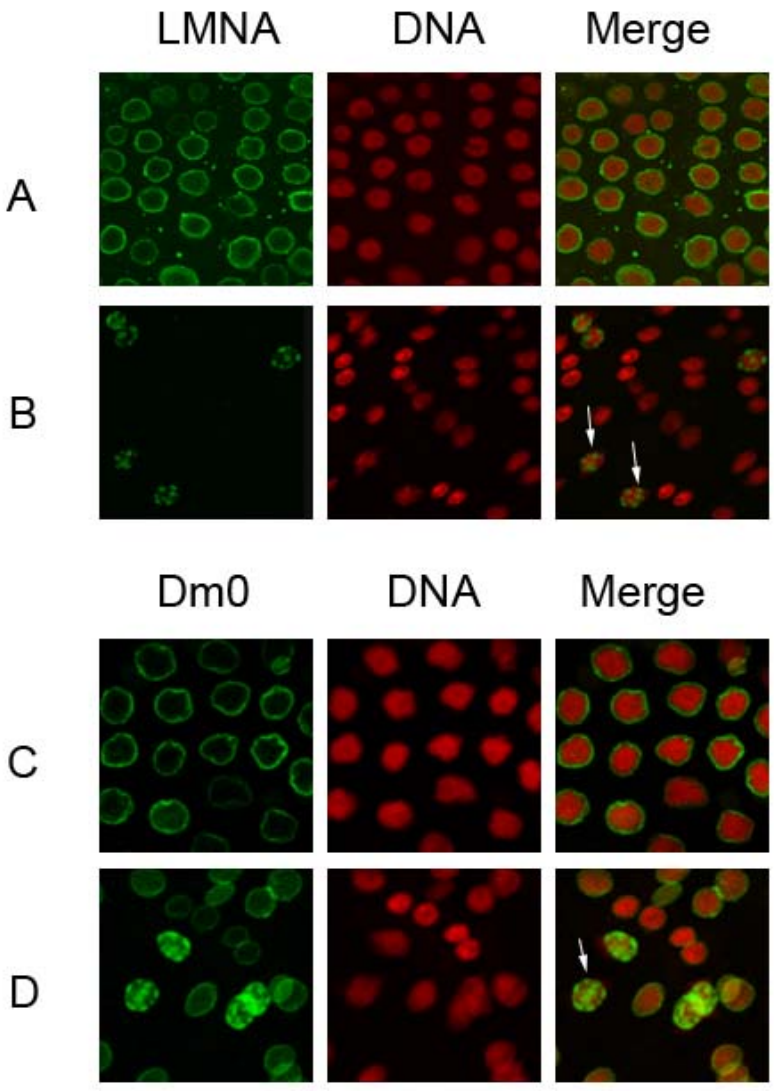


Figure C.2 Examples of ectopic expression of human LMNA in adult flies after heat shock treatment. Western blot analysis was performed on total protein extracts of *UAS-LMNA/CyO* or *UAS-LMNA/hsp70-GAL4* male flies 24 hours after 1-hr heat shock treatment. Results of two transgenic lines UAS-638.6a and UAS-638.4 were shown in the figure. Anti-LMNA antibody was used to detect human LMNA protein. Human LMNA protein was only detected in *UAS-LMNA/hsp70-GAL4* flies after heat shock treatment (arrow) but not in *UAS-LMNA/CyO* control flies. Weak signal detected in *UAS-LMNA/hsp70-GAL4* flies before heat shock induction is due to background leaky expression.

Figure C.3 Nuclear localization of lamin proteins in Drosophila male accessory gland. Accessory glands of *UAS-LMNA/hsp70-GAL4* male flies carrying the wild-type form of the human *LMNA* were dissected before or after heat shock induction. Glands were then fixed and stained with propidium iodide to visualize DNA (red) and with anti-LMNA or anti-lamin Dm_0 antibody to visualize lamin proteins (green). (A) In 19% (n=16) of the glands, *LMNA* staining was observed at the nuclear periphery. (B) In 81% of the glands, *LMNA* aggregated at nuclear periphery (arrows). (C) In 25% (n=12) of the glands tested, endogenous Drosophila Lamin Dm_0 was localized at the nuclear periphery and formed a ring-like structure. (D) In 75% of the glands tested, a nuclear aggregation of endogenous Drosophila Lamin Dm_0 was observed (arrows).


LMNA
DNA
Merge
A
B
Dm0
DNA
Merge
C
D

observed in salivary glands overexpressing a mutant form of the Drosophila LamC protein (Schulze et al., 2005). This aggregation is possibly the cause of the lethal phenotype when the transgenic human *LMNA* protein was overexpressed by the ubiquitous *tubulin*-GAL4 induction.

I also examined the nuclear localization of endogenous Drosophila B-type lamin (Lamin Dm_0) in flies overexpressing human *LMNA* protein. In 25% (n=12) of the glands tested, endogenous Drosophila Lamin Dm_0 was localized at the nuclear periphery and formed a ring-like structure (Figure C.3C), while in 75% of the glands tested, a nuclear aggregation of endogenous Drosophila Lamin Dm_0 was observed (Figure C.3D, arrow). This aggregation phenotype resembles the staining pattern of ectopically expressed *LMNA* proteins, which suggests that this phenotype is probably caused by the aggregation of overexpressed *LMNA*.

Summary: I have shown that human *LMNA* can be expressed in the flies using UAS-GAL4 expression system. Ubiquitous expression of the human *LMNA* during development causes lethality in the fly. Transient expression of the human *LMNA* protein in adult flies does not affect the viability of the fly. Ectopically expressed *LMNA* proteins are aggregated at the nuclear periphery.

APPENDIX D

IDENTIFYING MOLECULAR LESIONS IN PRAGE MUTANTS

D.1 Introduction

Embryos from *prage (prg)* mutant females cannot hatch into adult animals. These embryos were previously reported to be unable to destabilize maternal transcripts, a phenotype that suggested a defect in early embryogenesis (Brent et al., 2000; Tadros et al., 2003). I conducted deficiency mapping to determine the chromosomal localization of the *prg* gene. DNA sequencing was then used to identify the molecular nature of the *prg* mutations.

D.2 Materials and Methods

Drosophila stocks and complementation tests: prg^{16A}/FM6 and prg^{32}/FM6 (Tadros et al., 2003) were kindly provided by W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). Deficiency strains *Df(1)BSC719*/ Binsinscy, *Df(1)ED6565*/FM7h, *Df(1)A94*/FM6, *Df(1)BSC530*/Binsinscy, *Df(1)260-1*/FM4 and *Df(1)AD11*/FM7c were obtained from the Bloomington Drosophila Stock Center (Indiana) and used for complementation tests. I crossed 3-days old virgin females of each deficiency strain to prg^{16A} and prg^{32} males. *prg*/*Df* female progeny from these crosses were scored to test for complementation.

Genomic DNA sequencing: Whole fly genomic DNAs were extracted from prg^{16A} and prg^{32} males and used as template to amplify target regions using GoTaq PCR amplification kit (Promega, Madison, WI). Primers used for sequencing are listed in Table D.1. DNA sequencing was performed by Cornell Life Sciences Core Laboratories Center (Cornell University, Ithaca, NY).

Table D.1 Sequences of PCR primers.

Primer	Sequence 5' -> 3'
CG14801-1	GTTGCACCTGCGCAAAAGAA
CG14801-2	TTCCCGCAAGCCAGGAGTTA
CG14801-3	CAGCGAAAGAGTCGGCAACA
CG14801-4	ACGTATGTGGGCGGACGACT
CG14801-5	GTTGGGAAATTTCAGCGATCA
CG14801-6	GCTGACGCCATCGTAGTACCG
CG14801-7	GCCGGTGGAAAGGAGTCTGTC
CG14801-8	GTCCTCCTCCTCCTTCGCTTG

D.3 Results

The *prage* alleles carry nonsense mutations in CG14801: *prg* had been previously mapped to the polytene chromosome region 1B4-1E2 (Brent et al., 2000; Tadros et al., 2003). To further map the *prg* gene, I carried out complementation analysis with deficiencies lines available from the Bloomington Drosophila Stock Center. I tested for complementation between *prg* mutant alleles (prg^{16A} and prg^{32}) and six deficiencies in the 1B4-1E2. One line, *Df(1)BSC719*/Binsinscy, fails to complement both *prg* alleles, while another line, *Df(1)A94*/FM6, carrying a partially overlapping deficiency complements both alleles. These results suggest that *prg* mutation is in chromosome region to 2B12-13. Ten genes in these were sequenced in both *prg* alleles. As shown in Figure D.1, both *prg* alleles have molecular lesions in the predicted ORF of CG14801, according to the latest annotation of the Drosophila genome (http://www.flybase.org). Both *prg* alleles have single base pair changes in the 3' end of the transcript which are shared by all six isoforms. CG14801 is a putative exonuclease as its primary protein sequence has a conserved exonuclease domain. In both *prg* mutations, single coding codon changed to a stop codon results truncated proteins of 72 a.a. and 373 a.a., respectively. Both truncated proteins lack the exonuclease domain, which could lead to complete loss-of-function of the CG14801 protein.

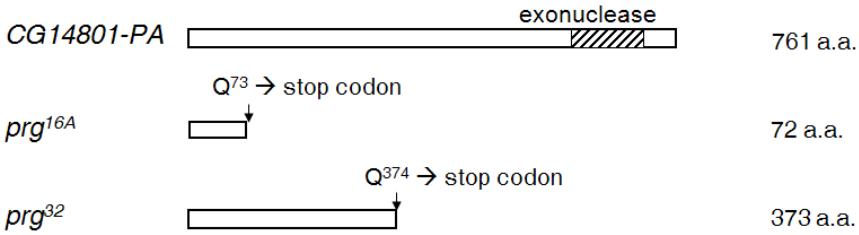


Figure D.1 Schematic representation of *prg* alleles. Wild-type CG14801 gene (isoform A) encodes a protein of 761 a.a. in length. *prg*^{16A} and *prg*^{32} have nonsense mutations in the coding region that result in truncated proteins of 72 a.a. and 373 a.a. respectively.

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